# METAGENOMIC CHARACTERIZATION OF POULTRY ENTERIC VIRAL AND BACTERIAL PATHOGENS AND GUT MICROBIOTA IN SELECTED REGIONS IN KENYA AND ASSOCIATED ANTIMICROBIAL RESISTANCE GENES

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# Metagenomic Characterization of Poultry Enteric Viral and Bacterial Pathogens and Gut Microbiota in Selected Regions in Kenya and Associated Antimicrobial Resistance Genes

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**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology of the Jomo Kenyatta University of Agriculture and Technology**

**2024**

## DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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## DEDICATION

This work is dedicated to my loving wife Joan, my children Elvis, Ryan, Leon, Boaz and Zawadi, and my parents, whose unconditional love, support, devotion, and encouragement have seen me through this journey of realizing my best self.

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## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **ALV** | Avian leukosis virus |
| **AMR** | Antimicrobial resistance |
| **ANV** | Avian Nephritis Virus |
| **APEC** | Avian Pathogenic *Escherichia coli* |
| **ARV** | Avian Reovirus |
| **ARGs** | Antimicrobial resistance genes |
| **BLAST** | Basic Local Alignment Search Tool |
| **bp** | Base pairs |
| **CAstV** | Chicken astrovirus |
| **ChPV** | Chicken parvovirus |
| **COG** | Cluster of Orthologous Genes |
| **DAstV** | Duck-origin astrovirus |
| **DEGs** | Differentially expressed genes |
| **DNA** | Deoxyribonucleic acid |
| **ESBL** | Extended-spectrum beta-lactamase |
| **EU** | European Union |
| **F** | Fusion protein |
| **GIT** | Gastrointestinal tract |

| | |
|---|---|
| **HBV** | Hepatitis B virus |
| **HDV-like** | Hepatitis D virus-like |
| **HGT** | Horizontal gene transfer |
| **IBV** | Infectious Bronchitis Virus |
| **ITS** | Internal transcribed spacer |
| **KEGG** | Kyoto Encyclopedia of Genes and Genomes |
| **LPDV** | Lymphoproliferative disease virus |
| **M** | Matrix protein |
| **MAS** | Mal-absorption syndrome |
| **MDR** | Multidrug resistance |
| **MGE** | Mobile genetic element |
| **MOLFD** | Ministry of Livestock and Fisheries Development |
| **NGS** | Next Generation Sequencing |
| **NMDS** | Nonmetric multidimensional scaling |
| **NP** | Nucleoprotein |
| **NS** | Nonstructural protein |
| **ORF** | Open Reading Frame |
| **PBS** | Phosphate Buffered Saline |
| **PCA** | Principal Component Analysis |

| | |
|---|---|
| **PCoA** | Principal Coordinate Analysis |
| **PCR** | Polymerase Chain Reaction |
| **PEC** | Poult Enteritis Complex |
| **PEMS** | Poult Enteritis Mortality Syndrome |
| **RNA** | Ribonucleic Acid |
| **rRNA** | Ribosomal ribonucleic acid |
| **RSS** | Runting-Stunting Syndrome |
| **RT-PCR** | Reverse Transcription Polymerase Chain Reaction |
| **SCFAs** | Short Chain Fatty Acids |
| **STAMP** | Statistical Analysis of Metagenomic Profiles |
| **TAstV** | Turkey astrovirus |
| **TCoV** | Turkey coronavirus |
| **TuPV** | Turkey parvovirus |
| **TRV** | Turkey reovirus |
| **VP** | Viral protein |

## ABSTRACT

Indigenous backyard poultry, which is the most commonly reared poultry in Africa, is often raised fsdain a free-range production system that exposes them to a wide array of microorganisms due to their diverse diet. These microorganisms, including viruses, bacteria, archaea, and protozoa, colonize the host's gastrointestinal tract. Some of these gut microorganisms can be pathogenic or beneficial to the host. Additionally, the continued use/misuse of antimicrobial agents in animal production has led to the development of antimicrobial resistance against these antimicrobial agents. Metagenomics reveals novel and highly divergent pathogens and microbiota in the gut of poultry. Unfortunately, most metagenomic studies on poultry microbiomes have been carried out on poultry reared under controlled and regulated feeding regimes. This study characterized and detected enteric viral and bacterial pathogens in Kenyan poultry, evaluated poultry gut microbial community profiles, and detected the antimicrobial resistance genes in poultry in Kenya. A stratified, cross-sectional, purposive approach was used during sample collection. Cloacal swabs were collected from 599 birds (based on the Fisher equation) in Bungoma, Busia, Kilifi, Kwale, Nairobi, and Trans Nzoia for detecting enteric viral and bacterial pathogens in poultry and also investigating the presence of antimicrobial resistance genes. Faecal and caecal contents were also collected from 24 male and 24 female birds in Bungoma, Kilifi, Kwale, Siaya, and Turkana and used to profile the entire microbiota in the poultry samples and also determine antimicrobial resistance genes. DNA and RNA were extracted from cloacal samples and sequenced using the Illumina Miseq platform. The whole genome shotgun sequences were then analyzed using bioinformatics and statistical tools. DNA was also extracted from the faecal and caecal samples and sequenced using the Illumina Miseq platform. Thereafter, the whole genome shotgun sequ-ences were analyzed using statistical and bioinformatics analyses. The most abundant viral families were *Coronaviridae* (43.4%), *Reoviridae* (36.6%), and *Retroviridae* (11.4%). The study also demonstrated the presence of several viral pathogens, with the most abundant being Infectious bronchitis virus (42.6%), Rotavirus (35.0%), Reticuloendotheliosis virus (6.0%) and Lymphoproliferative disease virus (2.7%), thus providing important insights into the prevalence and diversity of pathogenic enteric viruses in poultry in Kenya. Additionally, the results indicate the presence of several potentially pathogenic bacteria, including *Chlamydiae* (11.38-98.43%) and *Proteobacteria* (1.57-85.46%) which were the most frequently detected phyla in the chicken cloacal samples, and *Proteobacteria* (28.51-100%) and *Firmicutes* (3.13-100%) which were the most frequently detected in other poultry. Furthermore, the study elucidates the presence of many commensals and potentially pathogenic microorganisms in the faecal and caecal contents of poultry. *Bacteroidetes* (1.60-93.48%), *Firmicutes* (3.22-48.40%), *Proteobacteria* (2.57-34.64%), and *Euryarchaeota* (0.32-22.72%) were the most frequently detected phyla in faecal samples, while *Bacteroidetes* (3.31-90.85%), *Firmicutes* (12.90-67.28%) and *Proteobacteria* (1.18-85.93%) were the most frequently detected phyla in caecal samples. Finally, the abundance of tetracycline, aminoglycoside, β-lactamase, and sulfonamide-resistance genes in most of the poultry analyzed raises concern about the dangers associated with continuous and inappropriate use of these antimicrobials in poultry production. These results provide

useful information for managing viral and bacterial diseases while broadening our understanding of the poultry gut microbiome in indigenous backyard poultry. Additionally, the antimicrobial resistance genes data provides a valuable indicator of the use of antimicrobials in poultry by smallholder backyard farmers in Kenya.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background Information

### 1.1.1 Trend in Poultry Production

Poultry are domesticated birds mainly kept by humans for their eggs, meat, and/or feathers. They include birds belonging to the orders *Galliformes* (chickens, quails, turkeys, and guinea fowls), *Anseriformes* (domestic ducks and geese), and other domesticated birds such as pigeons. The domestic-cation of poultry started over 2,000 years ago (Ogali, 2020). Poultry farming has since continued to increase, making it an important industry that accounts for ~ 40% of the total meat consumed globally as of 2020 (Abreu et al., 2023).

Poultry farming is widely practiced because of its economic importance, being amongst the most extensive food sectors across the world. Poultry meat and egg production is a source of livelihood for farmers and a major protein source for consumers. Currently, poultry meat is the most widely consumed meat type globally. The largest poultry producers are China (28 %), the United States (12 %), and Brazil (7 %) (FAO, 2022). Among poultry, chickens are often the most farmed, with extensive production of their meat; more than 90 billion tons annually (FAO, 2022).

In Kenya, the most common poultry reared include chickens, ducks, guinea fowls, quails, geese, turkeys, pigeons, and ostriches. Kenya has an estimated poultry population of 43.8 million birds, dawith indigenous chicken forming the largest proportion (75%), while layers and broilers constitute 24% of the poultry population (Government of Kenya, 2019). Other poultry like ducks, geese, turkeys, pigeons, ostriches, guinea fowls, and quails make up less than 2 % of the poultry pr-oduction (Government of Kenya, 2019). Indigeneous poultry therefore comprises the bulk of poultry stocks in low-income, food-deficit countries (about 80%), and considerably contributes to improving human nutrition, generating income, and providing manure for crop production.

### 1.1.2 Poultry Production Systems

Three methods are adopted for rearing poultry; free range system, semi-intensive system, and intensive system. The free-range system is mainly used by less endowed farmers and represents the principal method of rearing poultry in Africa (AU-IBAR, 2016). In the intensive system, poultry do not have access to an outdoor enclosure. They are kept confined in cages (also referred to as the battery system). The system requires proper housing, equipment, and skilled labor ((National Research Council, 1991; AU-IBAR, 2016).

In Kenya and other developing countries, poultry species are mainly reared under free-range (scavenging) systems in rural settings, hence, commonly referred to as village poultry (Khobondo, 2015). This system is preferred because it is less capital-intensive and applies minimal biosecurity measures (Nyaga, 2007). Additionally, indigenous poultry is known to be more tolerant to diseases such as Newcastle, infectious bursal disease (gumboro), and salmonellosis than commercial chicken.

### 1.1.3 Challenges in Poultry Production

The demand for poultry products such as meat and eggs has pushed many farmers to intensify poultry production over the last century, resulting in the industry's rapid growth. The current poultry biomass, for instance, accounts for about 70% of the total biomass of birds globally (Bar-On et al., 2018; François & Pybus, 2020). In many intensive systems, poultry flocks are often kept in high-density populations that are genetically homogeneous. This potentially makes them susceptible to outbreaks of infectious diseases, leading to substantial economic losses and food insecurity (François & Pybus, 2020). The gastrointestinal tract (GIT) of poultry has been identified as a reservoir of many poultry pathogens and commensal microorganisms, making it a critical study area.

Enteric diseases affecting poultry are economically important because they directly decrease feed absorption, resulting in retarded growth, poor feed conversion leading to increased production costs, immunosuppression, and increased mortality arising from secondary infections (Koo et al., 2013). Many viruses, including astroviruses,

reoviruses, rotaviruses, coronaviruses, and adenoviruses which have been implicated as etiological agents of enteric diseases, have been isolated from or identified in the intestines or intestinal contents of infected poultry (Pantin-Jackwood et al., 2008).

Several enteric bacterial infections also pose a serious threat to gut health, such as necrotic enteritis caused by toxigenic *Clostridium perfringens*, ulcerative enteritis caused by *Clostridium colinum*, colibacillosis caused by *Escherichia coli*, erysipelas caused by *Erysipelothrix rhusiopathiae*, and fowl cholera caused by *Pasteurella multocida* (Porter, 1998). Additionally, the intestinal tract is prone to *Salmonella* colonization and transmission. *Salmonella*-induced enteric poultry lesions are often associated with three diseases; pullorum disease, fowl typhoid, and paratyphoid infections. Furthermore, both pathogenic and nonpathogenic *Spirochetes* also inhabit poultry guts (Porter, 1998).

The poultry gut is exposed to exogenous microorganisms immediately after hatching, and thereafter, it becomes a warm shelter for a plethora of microbiota (Pan & Yu, 2014). Microbiota in the GIT play an important role in the host's health, such as nutrient absorption, feed digestion, and immune system modulation (Mohd Shaufi et al., 2015). Enteric microbes are also involved in reducing and preventing colonization by enteric pathogens through competitive exclusion and production of bacteriostatic and bacteriocidal substances (Clavijo & Flórez, 2018). Despite these benefits, the poultry gut microbiota also acts as a source of human infections and a reservoir of antibiotic-resistance determinants (Sergeant et al., 2014). It has also been elucidated that an optimal gut microbiota can enhance agricultural productivity, as reported by Huyghebaert et al. (2011). Furthermore, gut microbiota also harbours many important genes of scientific interest and biotechnological potential (Sergeant et al., 2014). Due to the need to understand the interplay between gut microbiota, poultry productivity, and observed disease signs, a repository of information about the complex gut microbiota is required.

Additionally, there is growing concern about the extensive use of antimicrobial agents in poultry production. These antimicrobial agents are usually administered in the feed or drinking water of poultry (Gyles, 2008) and used for growth promotion,

disease prophylaxis, and treatment. More worrying is the trend where whole herds and flocks are treated with antimicrobial agents to prevent diseases and promote growth in livestock (Witte, 1998; van den Bogaard & Stobberingh, 1999; Yang et al., 2004). Ocassionally, sick poultry are treated individually, but oftenly, whole flocks are treated at once, including those that are not ill (Yang et al., 2004). Such inappropriate and unregulated use of antimicrobial agents increases the likelihood of selecting organisms that are resistant to the antimicrobial agent. Of foremost concern is the emergence of resistance to frontline antibiotics such as fluoroquinolones, which are very vital for treating human infections because of their low toxicity and broad spectrum coverage (Angulo et al., 2000; Livermore et al., 2002; Yang et al., 2004). In addition to human health concerns, antimicrobial-resistant pathogens also pose a major challenge to animal health due to higher morbidity and mortality (Yang et al., 2004). Unfortunately, most studies that have characterized antimicrobial resistance in Kenyan poultry have employed the cumbersome culturing techniques (Nguyen et al., 2016). There is therefore need to delve deeper into the prevalence of antimicrobial resistance in Kenyan poultry using the more advanced next-generation sequencing platforms.

### 1.2 Statement of the Problem

Poultry enteric viral and bacterial diseases are of significant economic importance, with financial losses to affected hatching egg producers and hatcheries estimated at 105,000 US dollars or 68,000 US dollars per 10,000 hens, respectively (Adebiyi et al., 2019). Additionally, the benefits of gut microbiomes to the host, such as providing nutrients from otherwise poorly utilized dietary substrates, modulating the development and function of the digestive and immune system, and reducing and preventing colonization by enteric pathogens, have not been adequately reported in Kenya. Furthermore, most metagenomic studies on poultry microbiomes have been carried out on poultry reared under controlled and regulated feeding regimes (Kumar et al., 2020). Paradoxically, while the gut microbiomes exert numerous health benefits, the high density of microorganisms within these ecosystems also facilitates the horizontal transfer of antimicrobial resistance genes to potential pathogenic bacteria (Zhou et al., 2012). AMR has thus gradually increased over the last few

decades, and currently accounts for almost 7 million deaths per year globally, which is estimated to increase to 10 million by the year 2050; with 90% of these deaths in low and middle income countries of Africa and Asia (Habiba et al., 2023). Previous molecular studies of viral and bacterial pathogens, gut microbiota, and AMR mainly focused on culture and PCR-based methods depended on the cultivability of the microbe and prior knowledge of the genome sequences of the said pathogens, whose presence in samples could then be investigated (Qiu et al., 2019). Hence, unidentified agents would never be encountered. Metagenomics reveals novel and highly divergent pathogens, microbes, and AMR determinants in the gut of poultry. There is therefore need to characterize and detect enteric viral and bacterial pathogens in Kenyan poultry, evaluate the poultry gut microbial community profiles, and detect associated antimicrobial resistance genes in poultry raised under free-range production systems in Kenya.

### 1.3 Justification

Poultry farming is amongst the most extensive food sectors across the world, with extensive production of poultry meat (especially chicken); more than 90 billion tons annually (Food and Agriculture Organization of the United Nations, 2017). Enteric viral and bacterial pathogens are of major economic importance in the poultry industry because they directly decrease feed absorption leading to growth retardation, increased production costs, immunosuppression, and sometimes increased mortality due to secondary infection (Mohd Shaufi et al., 2015). It is therefore crucial to characterize these pathogens in Kenya to better diagnose and manage them for increased production. Intensive selection over decades has also produced poultry breeds that convert feed into muscle mass (high-quality protein) efficiently. Extraction of energy and nutrients requires interaction between poultry biochemical functions and the microbiota present in the GIT. The selection of beneficial microbiota plays an important role in the production, health, protection from enteric pathogens, detoxification, and modulation of the immune system (Wu and Wu, 2012). There is also need to reduce the overreliance on antimicrobial agents in poultry production as they lead to increased AMR. Comprehensive analysis of the gut microbiota is therefore important to better understand the microbial interactions

and biodiversity, which is important for implementing strategies to improve gut health. Additionally, most metagenomic studies on poultry microbiomes have been carried out on poultry reared under controlled and regulated feeding regimes (Kumar et al., 2020). Metagenomic analysis of poultry raised under a free-range feeding system is thus crucial in understanding the dynamics of transmission of antimicrobial resistance and the evolution of populations of enteric pathogens resistant to antimicrobial agents. This will ultimately help in designing programs aimed at the prudent use of antimicrobial agents and reducing antimicrobial resistance.

## 1.4 Research Questions

1. Are poultry in Kenya affected by enteric viral pathogens?
2. Are poultry in Kenya affected by enteric bacterial pathogens?
3. Are there significant differences in enteric microbial profiles of poultry in Kenya?
4. Are enteric pathogens in Kenyan poultry resistant to antimicrobial agents?

## 1.5 Objectives

### 1.5.1 General Objective

To detect and characterize enteric viral and bacterial pathogens, evaluate gut microbial profiles, and determine antimicrobial resistance genes in poultry in selected regions in Kenya.

### 1.5.2 Specific Objectives

1. Characterize enteric viral pathogens in Kenyan poultry
2. Characterize enteric bacterial pathogens in Kenyan poultry
3. Evaluate gut microbial community profiles of poultry in Kenya.
4. Determine antimicrobial resistance genes in Kenyan poultry.

## 1.6 Null Hypotheses

1. There are no enteric viral pathogens in Kenyan poultry.
2. There are no enteric bacterial pathogens in Kenyan poultry.
3. There are no significant differences in enteric microbial community profiles of poultry in Kenya.
4. Enteric pathogens in Kenyan poultry are not resistant to antimicrobial agents.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction

The demand for poultry products such as meat and eggs has encouraged many farmers to intensify the production of poultry over the last century, resulting in the rapid growth of the industry. Poultry flocks are often kept in high-density populations that are genetically homogeneous. This potentially makes them vulnerable to outbreaks of infectious viral and bacterial diseases, leading to significant economic losses and food insecurity (François & Pybus, 2020). Moreover, many important pathogens of domesticated birds are shed and disseminated by wild birds (François & Pybus, 2020). Additionally, some zoonotic viral pathogens of pandemic potential have been known to originate from poultry and other domestic livestock such as pigs and horses (Amimo et al., 2016). Furthermore, despite the importance of the gut in poultry, there is limited information about the complex gut microbial community (Day & Zsak, 2013). Additionally, there is now an urgent need to understand how misuse and/or inappropriate use of antimicrobial agents leads to an increased likelihood of selecting organisms that are resistant to some of these antimicrobial agents.

## 2.2 Characterization of Poultry Enteric Viral Pathogens

Different types of microorganisms, collectively called microbiome, are found in poultry. They include bacteria, archaea, fungi, and viruses. They are frequently introduced into their hosts through the GIT where they thrive (Day et al., 2015). Many studies on bacterial communities (bacteriome) have underscored their important role to the health of the host where they either establish a symbiotic, commensal, or pathogenic relationship (Ramírez-Martínez et al., 2018). Similarly, the viral community (virome) comprising eukaryotic viruses, bacteriophages, host viruses, and viral genetic elements integrated into the host's genome, also includes pathogenic viruses implicated in several host diseases (Ramírez-Martínez et al., 2018). Besides the pathogenic viruses, the focus is shifting toward the interactions

between viruses, their hosts, and other microbiomes, as well as the effects that persistent viruses have on immunomodulation and susceptibility to diseases (Ramírez-Martínez et al., 2018).

### 2.2.1 Common Enteric Viral Pathogens of Poultry

Poultry enteric viral pathogens are of major economic importance because they result in decreased weight gain, increased morbidity and mortality, and increased cost of production due to poor feed conversion and treatment. Some of these potentially pathogenic viruses include astroviruses, rotaviruses, coronaviruses, parvoviruses, retroviruses, among others.

Avian astroviruses are commonly found in the poultry gut, especially in combination with other enteric viruses, and these viruses are associated with enteric disease syndromes (Day & Zsak, 2013). Currently, there are five recognized avian astrovirus species: two of turkey origin, TAstV-1 and TAstV-2; two of chicken origin, avian nephritis virus (ANV) and chicken astrovirus (CAstV); and duck-origin astrovirus (DAstV). Avian astroviruses are reported to be able to cause gross enteric disease signs, such as diarrhea and mild bursal lesions in poults and turkey embryos (Nighot *et al*., 2010).

Avian rotaviruses were first described as the possible cause of enteritis in turkey poults by Bergeland *et al*. (1977). Since then, they have been reported in other avian hosts (Guy, 2008; McNulty & Reynolds, 2008; Day & Zsak, 2013). Rotaviruses are regularly detected in concomitant infections with other viruses such as astroviruses and reoviruses. However, rotaviruses and other coinfecting viruses are also frequently found in healthy flocks presenting no enteric disease signs (Day & Zsak, 2013). Evidence of interspecies transmission of rotavirus between chickens and turkeys (Schumann *et al*, 2009; Day and Zsak, 2013), between mammals and poultry (Brussow *et al*., 1992a; Brussow *et al*., 1992b; Rohwedder *et al*., 1995; Wani *et al.*, 2003; Asano *et al.*, 2011), and between wild and domestic birds (Marlier & Vindevogel, 2006; Ursu *et al*., 2011) has been reported. Increased surveillance for rotavirus in wild birds and agricultural animals is therefore necessary to understand the prevalence and molecular epidemiology of avian rotaviruses (Day & Zsak, 2013).

Coronaviruses, especially members of the genus *Gammacoronavirus,* have been implicated as the aetiologic agents of contagious viral enteritis in turkeys (blue comb disease or mud fever) (Day and Zsak, 2013). Coronaviruses have also been reported in other avian species, for instance, turkey coronavirus (TCoV) which can cause high mortality in poults when a coinfection of a PEMS-associated enteropathogenic *Escherichia coli* strain R98/5 is present (Guy, 2000 Day and Zsak, 2013), and infectious bronchitis virus, which is a common viral pathogen of poultry.

Parvoviruses have also been implicated as causative agents of enteric disease in poultry. Some of the common poultry parvoviruses include the chicken and turkey parvoviruses. Most of the parvovirus-positive flocks experience enteric disease syndromes, with commercial chickens and turkeys being the only natural hosts where parvovirus infections have been observed (Day and Zsak, 2013). Broilers are especially susceptible to parvovirus infections and show symptoms of the clinical disease.

Avian reoviruses are also an important cause of enteric disease in chickens and turkeys and are involved in myocarditis, viral arthritis, infectious tenosynovitis, mal-absorption syndrome, and respiratory and enteric diseases (Jindal *et al*., 2014). The reoviruses detected in turkeys are commonly called turkey reoviruses (TRV) as opposed to ARV in chickens (Jindal et al., 2014). The TRV is often isolated from cases of turkey enteritis and tenosynovitis, resulting in substantial economic losses to turkey farmers (Jindal *et al*., 2014).

### 2.2.2 Approaches to Studying Enteric Viral Pathogens

Several approaches have been employed to study viral pathogens in poultry. Previous methods involved extracting and subsequently amplifying the viral nucleic acids using conventional polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), and/or real-time RT-PCR (Qiu et al., 2019). Using this strategy, several viruses have been implicated in poultry diseases, including astroviruses (Day *et al*., 2007; Pantin-Jackwood et al., 2008; Pantin-Jackwood et al., 2011), parvoviruses (Koo et al., 2015), reoviruses (Chen et al., 2014), rotaviruses (Spackman et al., 2010), paramyxoviruses (Ogali et al., 2018), and coronaviruses (Chamings et al.,

2018; Woo et al., 2012). These methods often require prior knowledge of the target viral pathogen, which poses a challenge when investigating multiple pathogens in one or more hosts, especially from different viral families. Additionally, these methods are unable to monitor and provide an early warning system for pathogens of other poultry diseases as well as emerging viruses that have not yet been reported (Qiu et al., 2019).

Currently, metagenomics-based detection methods are becoming more popular for characterizing viral populations in different hosts and environments (Lima et al., 2019). This has expedited the discovery of a large number of novel viruses from different types of tissues, including the GIT of poultry and other livestock (Amimo et al., 2016; Day et al., 2010, 2015; Kim et al., 2012; Vibin et al., 2020). The attractiveness of these techniques has largely been due to their high sensitivity and wide coverage as they target the entire genome (Qiu et al., 2019; Vibin et al., 2020). They can therefore be used to detect all pathogens found in the sample of interest. Furthermore, these methods can discover novel viruses whose sequence information is unknown. Hence the genotypes, virulence, and molecular evolution of pathogens can be inferred directly using metagenomics analyses.

### 2.3 Characterization of Poultry Enteric Bacterial Pathogens

Bacterial pathogens cause diseases in both animals and humans, thus posing a threat to animal production, food safety, and public health (Zhou et al, 2012).

#### 2.3.1 Common Enteric Bacterial Pathogens of Poultry

Several bacterial pathogens affect poultry, for instance, *Salmonella*, *Campylobacter*, avian pathogenic *Escherichia coli*, and enterococci (Gyles, 2008).

##### 2.3.1.1 Clostridium

*C. perfringens* and *C. colinum* are Gram-positive spore-forming anaerobic bacteria that are widespread in the environment, especially in soil, sewage, and the gastrointestinal tract of animals and humans as members of the normal gut microbiota (Cooper and Songer, 2009; Razmyar *et al*., 2014). *C. perfringens* causes

histotoxic and enteric infections in humans and animals by producing a large number of toxins (Popoff and Bouvet, 2009; Razmyar *et al*., 2014). Necrotic enteritis is primarily caused by *C. perfringens* type A and to a lesser extent type C strains (Razmyar *et al*., 2014). *C. colinum* causes ulcerative enteritis, which is a highly fatal disease that mainly affects captive quail, although it has also been reported in other birds, including chickens, turkeys, and pheasants (Porter, 1998). Young quail, from 4-12 weeks old are most susceptible.

#### 2.3.1.2 Avian Pathogenic Escherichia Coli (APEC)

Although normally commensal in nature, certain strains of *Escherichia coli* are associated with a variety of infections in poultry (Yang *et al*., 2004). In chickens, they may cause infections of the respiratory tract and soft tissues, resulting in colibacillosis, air sacculitis, and cellulitis (Gross, 1991). *E. coli* is a Gram-negative, medium-sized (2 to 3μm long) rod that is widespread in nature and is a normal inhabitant of the intestinal tract of poultry (Gross, 1991). Pathogenic strains of *E. coli* can often be isolated from the intestinal tracts of healthy poultry, which supports the assertion that *E. coli* is often a secondary or opportunistic pathogen (Porter, 1998).

#### 2.3.1.3 Pasteurella Multocida

*P. multocida*, which is a small (<2μm long) Gram-negative nonmotile rod or coccobacillus that varies in virulence depending on the strain (Porter, 1998), is the aetiological agent of fowl cholera or pasteurellosis. Sixteen distinct strains, which are serotyped according to the type of surface lipopolysaccharide, have been reported (Brogden *et al*., 1978). Fowl cholera is most common in adult or young adult birds. Using PCR-based typing, multiple strains of *P. multocida* were detected in fowl cholera outbreaks in India (Shivachandra *et al*., 2005).

#### 2.3.1.4 Mycobacterium Avium

*M. avium*, the causative agent of avian mycobacteriosis (avian tuberculosis), is an acid-fast, nonmotile, aerobic rod that is long-lived in soil and dried faeces (Thoen *et al*., 1981). The disease affects a wide range of birds (Porter, 1998) with serovars 1

and 2 being the most commonly isolated from birds. *M. avium* is mainly transmitted via the faecal-oral route and has zoonotic potential, based on its isolation from humans with acquired immunodeficiency syndrome (Falkinham, 1994). *M. avium* has a long incubation period, the reason why the disease is most commonly diagnosed in adult birds (Porter, 1998). Avian mycobacteriosis in poultry is usually a chronic disease characterized by progressive loss of condition. Birds continue to lose body weight despite having normal feed consumption.

#### 2.3.1.5 Salmonella

The genus *Salmonella* contains over 2,000 serotypes that have been classified by somatic (O), flagella (H), and capsular (Vi) antigens (Porter, 1998). *Salmonella* is a Gram-negative, 2 to 3 µm long, nonsporulating rod that grows well on brilliant green and Mac Conkey agar. The intestinal tract is one of the most common sites of *Salmonella* colonization, and transmission is often by the fecal-oral route. *Salmonella* infections can be systemic (bacteremia) and are sometimes accompanied by enteric lesions. *Salmonella*-induced enteric lesions of poultry are often associated with three diseases; pullorum disease, fowl typhoid, and paratyphoid infections (Porter, 1998). In these diseases, the enteric lesions are often prominent in the caecum, which is the best site for bacteriologic isolation of enteric *Salmonella*.

Pullorum disease is caused by *S. pullorum* and fowl typhoid is caused by *S. gallinarum*. These microorganisms cause systemic disease in a wide range of domestic poultry, including chickens, turkeys, ducks, and other gallinaceous birds. Pullorum disease is most lethal in young birds (3 weeks or less) with minimal effects on adults, while fowl typhoid causes mortality in young birds and persists to adulthood (Pomeroy & Nagaraja, 1991). Birds recovering from pullorum disease and fowl typhoid become chronic carriers and transmit the infection to progeny through the eggs (transovarian transmission), with the infected progeny readily spreading the infection horizontally through contaminated faeces (Porter, 1998).

Most *Salmonella* serotypes are placed in the paratyphoid group, described as the *Salmonella* serotypes other than *S. pullorum*, *S. gallinarum*, and *S. arizona* (Ashton, 1990). The paratyphoid *Salmonella* are motile and infect a wide variety of host

species, and include *S. typhimurium*, *S. enteritidis*, *S. montevideo,* and *S. heidelberg*. Paratyphoid infections can be transmitted through the faecal-oral route, faecal soiling of eggshells, and contaminated feed. Mice are a common reservoir of paratyphoid infections. *S. enteritidis* is distinct among paratyphoid infections by being egg-transmitted via deposition into the egg yolk before lay, resulting in the production of *S. enteritidis*-contaminated eggs (Gast & Beard, 1990). Intestinal colonization by paratyphoid *Salmonella* normally results in invasion of the gut and dissemination to internal organs (Brown *et al*., 1976). Paratyphoid infections in young birds often result in systemic infections with high mortality. Birds may die without showing signs or may appear depressed with closed eyes, ruffled feathers, discoloured yolk sacs, and profuse diarrhoea. Adult birds appear to be rather resistant to paratyphoid infections and may harbor *Salmonella* in soft tissues without showing clinical signs (Brown *et al*., 1976). Adult hens infected with *S. enteritidis* appear healthy and continue to shed the pathogen in the faeces (Holt & Porter, Jr, 1993).

#### 2.3.1.6 Spirochaetes

Spirochaetes are slender, motile, flexible, unicellular, and helically coiled bacteria ranging from 0.1 to 3.0 µm in width (Quinn *et al*., 1994b). Both pathogenic and nonpathogenic spirochaetes appear to inhabit the intestinal tracts of birds (Buckles *et al*., 1997). Two genera of spirochaetes are associated with enteric disease in poultry. One is a weakly β-haemolytic spirochaete in the genus *Serpulina* which is associated with infectious typhlitis in commercial laying hens and broiler chickens (Dwars *et al*., 1992; Swayne *et al*., 1992). The other one is the chicken-origin spirochaete that mainly infects chickens. The chicken-origin spirochaetes are also weakly β-haemolytic with alpha-galactosidase surface antigens and do not produce indole (Porter, 1998). The chicken-origin spirochaetes have been isolated from the intestine of birds with retarded growth, diarrhoea, faeces-stained egg shells and vents, and decreased egg production (Dwars *et al*., 1992; Swayne *et al*., 1992; Porter, 1998). Gross lesions are often limited to the caecum which contains a light brown, frothy liquid (Trampel *et al*., 1994).

### 2.3.2 Metagenomics as an Approach for Characterizing Enteric Pathogens in Poultry

Metagenomic analysis has become the tool of choice for studying microbial communities and their importance in various environments, including the gastrointestinal tracts of animals. This approach allows for the identification of both cultivable and non-cultivable microorganisms and their associated genes, thus providing a more comprehensive picture of the microbial ecology of poultry (Pérez-Cobas et al., 2020).

Several studies have investigated the prevalence of bacterial pathogens in poultry in different parts of the world. For instance, Havelaar et al. (2015) estimated that the global burden of foodborne illness due to non-typhoidal *Salmonella* in poultry was over 60 million cases yearly. Unfortunately, limited studies have applied metagenomic characterization to investigate bacterial pathogens present in poultry raised in free-range environments in Kenya.

## 2.4 Analysis of Poultry Gut Microbiomes

The gastrointestinal tract of poultry is densely populated with microorganisms that closely and intensively interact with the host and ingested feed (Pan and Yu, 2014). The gut microbiomes have numerous benefits to the host, such as providing nutrients from otherwise poorly utilized dietary substrates, modulating the development and function of the digestive and immune systems, and reducing and preventing colonization by enteric pathogens through competitive exclusion and production of bacteriostatic and bacteriocidal substances (Pan and Yu, 2014; Clavijo and Florez, 2018). In return, the host provides a permissive habitat and nutrients for microbial colonization and growth. A comprehensive understanding of poultry gut microbiomes and their interactions with the host aid in developing new interventions that can enhance poultry growth, maximize host feed utilization, and offer alternatives to antimicrobial agents, which will ultimately protect birds from enteric diseases caused by pathogenic microbes.

### 2.4.1 Cultivation-Based Analysis of Poultry Gut Microbiota

Conventional methods for detecting microorganisms largely depended on isolation, identification, and differentiation of these microbes by employing serotyping and biochemical tests (Shivachandra *et al.*, 2005). However, these techniques are laborious and time-consuming, in addition to other limitations.

Early cultivation-based studies revealed low abundances of lactobacilli and clostridia in the small intestines and a high abundance of anaerobic bacteria in the cecum of chickens (Barnes *et al.*, 1972; Salanitro *et al.*, 1974). In the studies by Barnes *et al.* (1972) and Salanitro *et al.* (1974), the bacteria that were identified included anaerobic Gram-negative cocci, facultative anaerobic cocci, and streptococci. The major genera recovered from cecum by cultivation included *Peptostreptococcus*, *Propionibacterium*, *Eubacterium*, *Bacteroides*, and *Clostridium* (Barnes *et al.*, 1972). Another study utilizing the culture-based methods by Bedbury and Duke (1983) revealed abundance of *Eubacterium*, *Lactobacillus, Peptostreptococcus, Escherichia coli*, *Propionibacterium,* and *Bacteroides*. Although only revealing a limited number and diversity of bacteria, these earlier studies laid the foundation for microbiological studies of the intestinal microbiome in poultry.

### 2.4.2 16S rRNA Gene-Targeted Analysis of Poultry Gut Microbiome

DNA-based techniques are rapid, specific, and highly sensitive (Shivachandra *et al.*, 2005). Sequencing of 16S rRNA genes by first the Sanger sequencing technology and recently by the next-generation sequencing (NGS) technologies have allowed the comprehensive characterization of the intestinal microbiomes of poultry, with the sequence information greatly expanding our knowledge on the microbial diversity existing in the intestinal tract, particularly the cecum of chickens and turkeys (Wei *et al.*, 2013; Pan and Yu, 2014).

Through phylogenetic and statistical analysis of 16S rRNA gene sequences recovered from the intestinal microbiome of both chickens and turkeys, Wei *et al.* (2013) found 13 phyla of bacteria, with *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* accounting for over 90% of the intestinal bacteria of chickens and

turkeys. The most predominant genera found in both chicken and turkey were *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides*, albeit with different distributions between the two poultry species. This study analyzed the intestinal microbiome of indigenous chickens, ducks, guineafowls, geese, pigeons, and turkeys raised under free-range environments in Kenya.

### 2.4.3 Metagenomic Analysis of Poultry Gut Microbiome

Metagenomic analysis has become crucial for studying microbial communities and their importance in various environments, including the gastrointestinal tracts of animals. This approach allows for the identification of both cultivable and non-cultivable microorganisms and their associated genes, thus providing a more comprehensive picture of the microbial ecology of poultry (Pérez-Cobas et al., 2020). However, only a few studies have applied metagenomic characterization to investigate the microbial communities present in poultry raised in free-range environments in Kenya.

Most metagenomic studies on poultry microbiome have been carried out on poultry reared under controlled and regulated feeding regimes (Bhogoju et al., 2018; Andreani et al., 2020; Schreuder et al., 2021; Williams & Athrey, 2020; Kang et al., 2021). However, metagenomic studies on free-ranging poultry are more informative than those on poultry raised under controlled conditions (Kumar et al., 2020). This is because free-ranging poultry are exposed to a broader range of environmental conditions, which can influence their microbial communities (Kumar et al., 2020). In contrast, poultry raised under controlled conditions are exposed to a more homogenous environment, which may limit their microbial communities' diversity (Kumar et al., 2020). However, limited studies have investigated the microbial community profiles of indigenous backyard poultry raised under a free-range feeding system (Kumar et al., 2020). Metagenomics analysis of poultry raised under a free-range feeding system is therefore required to explore the impact of local feed (plants, insects, and other small animals) on poultry health (Kumar et al., 2020). In addition, this aids in understanding the microbiome compositional structure about the environment in free-ranging poultry. It is therefore necessary to characterize

microorganisms present in indigenous backyard poultry in Kenya using a metagenomic approach. This will aid in understanding gut microbiota of poultry raised in free-range environments better and also inform on the interventions needed to reduce the risk of foodborne illnesses.

### 2.5 Analysis of Antimicrobial Resistance Genes in Poultry

Antimicrobial agents are used extensively in poultry production and are usually administered in the feed or drinking water (Gyles, 2008). The use of antimicrobial agents has been proposed to be a contributor to the transformation of the poultry industry from large numbers of small-scale farmers to a small number of large-scale producers who operate at high efficiency (Bywater, 2005). Antimicrobial agents are used for growth promotion, disease prophylaxis, and treatment (Gyles, 2008).

As has been pointed out earlier, continued misuse and/or inappropriate usage of antimicrobial agents increases the likelihood of selecting organisms that are resistant to the antimicrobial agent (Yang *et al.*, 2004). Of major concern is the emergence of resistance to frontline antimicrobial agents such as fluoroquinolones, which are extremely valuable for treating human infections because of their low toxicity and relatively broad-spectrum coverage (Angulo *et al.*, 2000; Livermore *et al.*, 2002; Yang *et al.*, 2004). In addition to human health concerns, antimicrobial-resistant pathogens also pose a severe and costly animal health problem because they prolong illness and decrease productivity through higher morbidity and mortality. Unfortunately, there is paucity of data on the prevalence of antimicrobial-resistant poultry pathogens in many developing countries, including Kenya.

#### 2.5.1 Trend in Antimicrobial Drug Use and Resistance in Poultry

Antimicrobial agents have been used widely since the 1950s in poultry to improve feed efficiency and growth (Torok et al., 2011). They are also used to reduce morbidity and mortality associated with poultry diseases (Torok et al., 2011). Globally, 73% of all antimicrobial agents available in most retail stores are utilized in food animals (Van Boeckel et al., 2019). These antimicrobial agents are usually administered by injection or orally in feed and drinking water, with permitted uses

varying between countries and regions (Gyles, 2008). The use of antimicrobial agents for growth promotion in food animals continues worldwide, except in the European Union (EU) where it was banned in 2006, and in the U.S. where it was discontinued in 2017 (Roth et al., 2019). The various uses of antimicrobial agents in poultry production are shown below (Table 2.1).

**Table 2.1: List of Antimicrobial Agents and their Uses in Poultry Production**

| Antimicrobial use | Antimicrobial agent | Reference |
|---|---|---|
| Growth promoters | Zinc bacitracin | Gyles (2008) |
| | Procaine penicillin | Gyles (2008) |
| | Tylosin | Gyles (2008) |
| | Virginiamycin | Gyles (2008) |
| | Monensin | Gyles (2008) |
| Prophylaxis and treatment | Chlortetracycline | Yadav et al. (2016) |
| | Furazolidones | Oluwasile et al. (2014) |
| | Fluoroquinolones | Gyles (2008) |
| | Oxytetracycline | Yadav et al. (2016) |
| | Sulphonamides | Gyles (2008) |
| | Gentamycine | Yadav et al. (2016) |
| | Quinolones | Yadav et al. (2016) |

Excessive reliance on antimicrobial agents results in their unregulated use and/or abuse in human therapies, animal husbandry, and aquaculture (Yuan et al., 2019). This increases the likelihood of selecting organisms that are resistant to the antimicrobial agent (Yang et al., 2004). One of the major concerns is the emergence of resistance to frontline antimicrobial agents such as fluoroquinolones. These

antimicrobials are very valuable for treating human infections because of their low toxicity and broad-spectrum coverage (Yang et al., 2004).

Currently, it is widely accepted that antimicrobial resistance (AMR) is a major global public health concern responsible for over 2 million resistant infections and many deaths (Yuan et al., 2019). This is expected to get worse with the emergence and dissemination of multidrug-resistant 'superbugs' that can resist multiple antibiotic categories (McKenna, 2013; Yuan et al., 2019). Additionally, antimicrobial-resistant pathogens also pose a serious animal health problem because they prolong illness and decrease production through higher morbidity and mortality (Yang et al., 2004).

### 2.5.2 Transmission and Spread of Antimicrobial Resistance

Antimicrobial resistance is a natural and ancient phenomenon (Yuan et al., 2019). Production of antimicrobial agents is widely utilized by microbes to ward off competition between them (Yuan et al., 2019), and the synthesis of antimicrobial agents by microorganisms has evolved over millions of years (Yuan et al., 2019). Katale et al. (2020) conclude that the higher level of diversity of the AMR genes indicates that there might be several sources of resistant bacteria, or a flow of genes among various strains as a result of the transmission by mobile genetic components. Antibiotic producers must therefore naturally possess self-protection mechanisms to effectively escape the threats from competitors' antibiotics (Cordero et al., 2012; Wright, 2007; Yuan et al., 2019). This resistance can emerge through point mutations, re-assortment, or horizontal gene transfer (HGT) (Hoelzer et al., 2017).

Studies show that enteric pathogens and normal intestinal microbiota play a critical AMR development and transmission role under external stress via HGT (Witte, 1998; Zhou et al., 2012) which is mediated by mobile genetic elements (MGEs) such as plasmids, transposons, and integrons. This was also corroborated by studies showing that intestinal microbiomes may serve as reservoirs for antimicrobial resistance and spread of resistance to zoonotic pathogens (Fricke et al., 2009; Nandi et al., 2004; Oakley et al., 2014). Many of the well-recognized antimicrobial resistance genes (ARGs) are present in MGEs and can be transmitted to other bacteria of the same or different species (Yuan et al., 2019). Usually, ARG subtypes

associated with the same antibiotic class confer bacterial hosts with varying tolerance to this antibiotic (Yuan et al., 2019).

Wee et al. (2020) posit that livestock have been considered as the key reservoir for AMR that may spread to humans, and shared environmental edges and proximity with respect to livestock have been stated as the fundamental risk factors for transmitting AMR. According to the World Health Organization (WHO), antimicrobials applied in treating various infectious diseases in livestock might be similar to those applied for humans, leading to the spread and transmission of AMR in humans who are near these animals/livestock (Novais and Freitas, 2020). Additionally, the intensified application of other antimicrobials such as disinfectants and metals in the environment is considered a prospective factor for AMR selection (Novais and Freitas, 2020). As a result, the spread of variable and compound antimirocrobial resistant bacteria, MGEs, and genes across environmental, animal, and human sections is a composite process taking place through numerous channels, including food exposure, manure, wastewater, and many others, promoting the ongoing genetic exchange, evolution, and recombination of AMR features.

### 2.5.3 Methods Used to Study Antimicrobial Resistance Genes

Until recently, costs and less advanced techniques hampered the study of the AMR gene profiles of the entire microbiome of poultry (Penders et al., 2013). The antibiotic susceptibility of the indicator microorganisms was previously assessed by culturing the micro-organisms of interest and then subjecting them to antimicrobial sensitivity testing to determine the level of resistance to specific antimicrobial agents. The choice of the indicator microorganism(s) was mostly based on the clinical relevance of these organisms and their cultivability (Penders et al., 2013). The limitation of these methods is that less than 20% of enteric microbiota have been cultured to date due to the fussy nature of intestinal micro-organisms that often require unknown culture requirements (Clavijo and Flórez, 2018).

Several polymerase chain reaction (PCR) techniques have also been exploited for detecting antimicrobial resistance genes. These methods improved the sensitivity and speed of detection of antimicrobial resistance. The limitation to the utilization of

PCR is that results are often skewed towards known antimicrobial resistance genes and mechanisms (Penders et al., 2013). It is also not possible to adopt these techniques to the assessment of the entire resistome in the gastrointestinal tract of poultry (Clavijo and Flórez, 2018).

Several studies utilizing PCR techniques have been conducted to characterize AMR in poultry. A study of small-scale and backyard chickens in Kenya showed a high rate of resistance of *Campylobacter jejuni* isolates to nalidixic acid, tetracycline, and ciprofloxacin (Nguyen et al., 2016). A lower resistance was also detected for gentamicin and chloramphenicol, with multidrug resistance detected in 61.3% of the isolates. Adelaide et al. (2008) also conducted a study to examine MDR and the existence of virulence-related genes in avian pathogenic *E. coli* isolates from broilers during the slaughter period in a processing plant in Kenya. The study found that there is a significant existence of MDR and virulent APEC amongst broilers in Kenya. In a recent study in Southern Ethiopia to determine sources and patterns of AMR in *Salmonella* isolated from the poultry industry, Abdi et al. (2017) showed that prevalence was higher in the bedding (35.3%) and personnel hand swabs (33.3%) than in the chicken cloaca (14.8%). This demonstrated the poor biosecurity and personnel hygiene practices in poultry handling centers. The authors also observed that all the isolates exhibited resistance to kanamycin and sulfamethoxazole-trimethoprim and showed varying resistance to nalidixic acid, ampicillin, cefoxitin, streptomycin, tetracycline, chloramphenicol, ciprofloxacin, and gentamicin. They were also alarmed to note that most isolates exhibited MDR. In yet another study investigating the occurrence, antimicrobial resistance, and virulence of *Enterococcus* spp. from poultry and cattle farms in Nigeria, all the isolates tested were susceptible to vancomycin. Resistance to tetracycline, erythromycin, ampicillin and gentamicin, however, was observed among 61%, 61%, 45.1%, and 32.7% of the isolates respectively (Ngbede et al., 2017). The authors also noted that 53.1% of the isolates were multidrug resistant. They detected antibiotic resistance (*tetK*, *tetL*, *tetM*, *tetO* and *ermB*) and virulence (*asa1*, *gelE* and *cylA*) genes among the isolates. From these observations, the authors inferred that poultry, cattle and manure in the study areas were hosts to varying *Enterococcus* species harbouring virulence and resistance determinants that could be transferred to other organisms.

Another important method for studying AMR is functional metagenomics. This involves the cloning of DNA fragments into a vector such as a plasmid that is subsequently expressed in heterologous hosts (often *E. coli*). The resultant transformants are then screened for the expression of resistance genes by growing them on antibiotic-containing media at concentrations where the wild-type host strain is susceptible. The antibiotic resistant clones are then subsequently sequenced (Penders et al., 2013). The main limitation of this method is that it depends on each gene's ability to be expressed in surrogate hosts. Resistance genes that are not expressed by the surrogate host are left unidentified (false negatives). At the same time, a foreign gene interacting in unique ways with the cellular machinery of the surrogate host results in false positives (Penders et al., 2013).

Sequence-based metagenomics, involving the extraction, fragmentation, size-separation and random direct sequencing of DNA from an environmental sample without the need for culturing, has also gained wide acceptance as one the most accurate methods for studying AMR. The sequences generated can then be compared to international sequence databases to identify antimicrobial resistance genes (Schmieder and Edwards, 2012). The transition from Sanger sequencing to next generation sequencing (NGS) platforms such as the Roche 454 sequencer, the Genome Analyzer of Illumina, the SOLiD system of Applied Biosystems, and the longer read Nanopore and PacBio technologies, has resulted in a significant drop in costs, which was accompanied by an increased number and size of metagenomic sequencing projects (Penders et al., 2013). Although most of the NGS technologies yield lower contiguous read lengths and require greater genome coverage, their high throughput reduces cost and number of sequencing runs. The most commonly used sequencing technique involves amplification and sequencing of the 16S rRNA gene (in case of bacteria) and/or internal transcribed spacer (ITS) region (in case of fungi) in the sample DNA. Direct shotgun sequencing of the DNA sample of the entire microbial community is also increasingly being exploited due to its high sensitivity, reproducibility, and coverage.

Several studies have investigated the prevalence of ARGs in poultry. For instance, Havelaar et al. (2015) estimated that the global burden of foodborne illness due to

non-typhoidal *Salmonella* in poultry was over 60 million cases yearly. Additionally, a study in Poland comparing the AMR gene profiles of farm animals exposed to antimicrobial treatment to those of wild animals that seemed not to be subjected to antimicrobial pressure revealed higher levels of AMR in farm animals than in wildlife (Skarżyńska et al., 2020). Furthermore, Skarżyńska et al. (2020) underscored the potential of wildlife in disseminating AMR. Unfortunately, few studies have applied metagenomics to investigate antimicrobial resistance genes (ARGs) present in poultry raised under free-range environments. One such study by Kumar et al. (2020) found an abundance of tetracycline-resistance genes in Ethiopian indigenous chicken raised under free-range conditions. Another study by Ndukui et al. (2022) also found a high prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in poultry in Kenya.

Using a metagenomic approach, this study therefore, detected and characterized enteric viral and bacterial pathogens in poultry raised under free-range production systems in Kenya, evaluated their gut microbial community profiles, and detected associated antimicrobial resistance genes. The cloacal, faecal, and caecal samples have been widely used for the detection of poultry pathogens and microbiota because most bacterial and viral infections and microbial colonization in birds are mainly through the fecal-oral route, making these regions a critical study area (Chaves Hernández, 2014; Nuradji et al., 2015). The study's findings contribute to a better understanding of gut bacterial pathogens and poultry gut microbiomes of poultry raised in free-range environments while also informing on the interventions needed to reduce the risk of antimicrobial resistance.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Introduction

This chapter discusses the materials that were utilized together with the methods followed in the study. The information provided includes the study design, study area, study clearance and permits of compliance information, sample size determination, sample collection procedure, and data analysis approach.

## 3.2 General Methodology

### 3.2.1 Study Areas

The study was conducted in smallholder poultry farms in Western Kenya, Turkana, and the Coastal region and live bird markets in Nairobi. The targeted regions included counties bordering Uganda (Siaya, Bungoma, Busia, Trans Nzoia, and Turkana), maritime borders (Kilifi and Kwale), and urban areas of Nairobi. The sampled subcounties with smallholder farms included Teso South and Funyula in Busia County; Kimilili, Kabuchai, Bungoma Central, and Mt. Elgon in Bungoma county; Rarieda in Siaya County; Kwanza in Trans Nzoia County; Turkana County; Malindi and Kilifi North in Kilifi County; and Matuga, Msambweni and Lungalunga in Kwale County (Figure 3.1). Live bird markets were also sampled in Burma and Maziwa markets in Nairobi County.

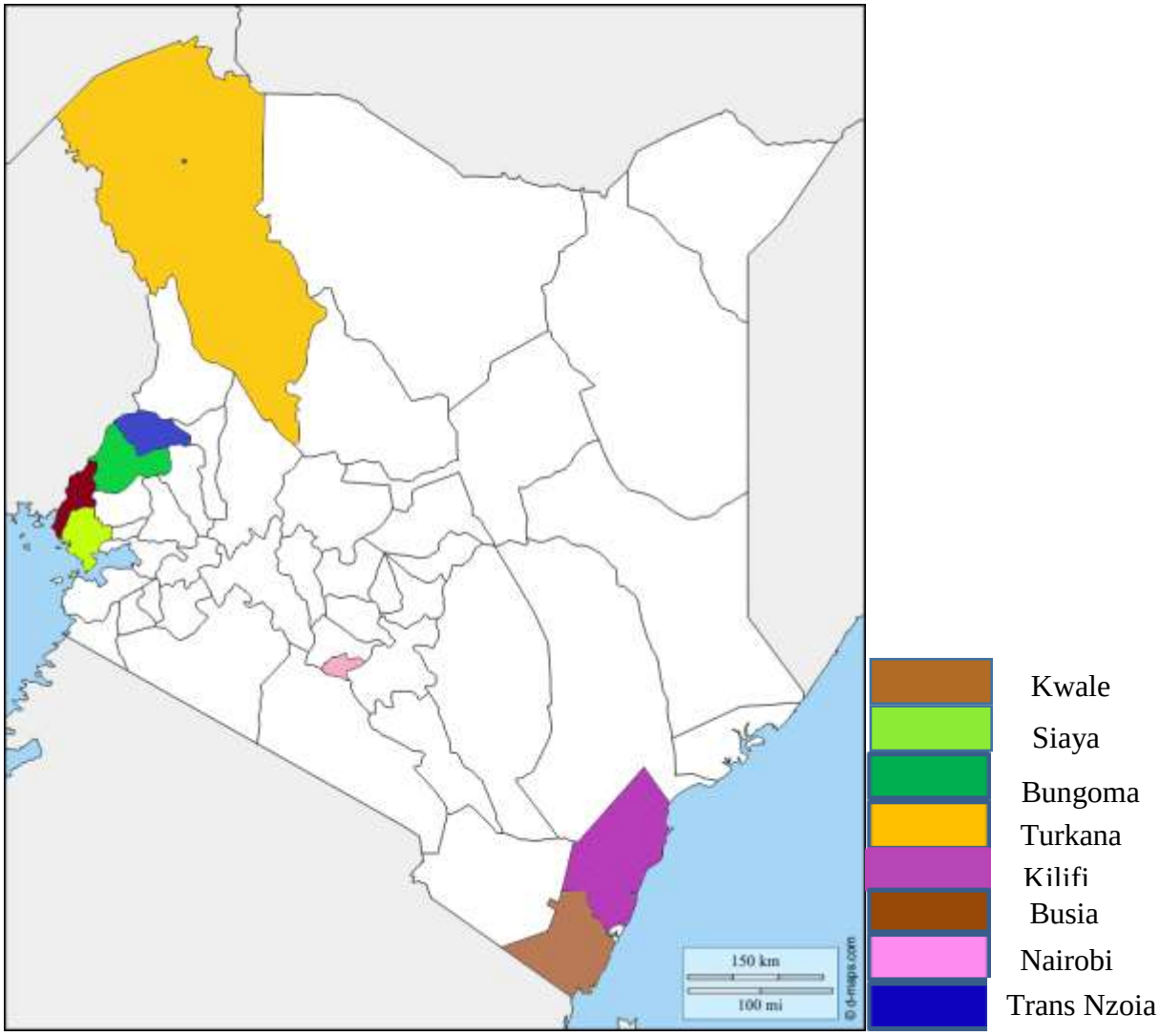


**Figure 3.1: Map of Kenya Showing the Main Sampling Sites** (*Source: D-Maps.Com*).

### 3.2.2 Description of the Study Areas

Western Kenya includes the regions of Busia, Bungoma, Siaya, and Trans Nzoia, while the Coastal region includes Kilifi and Kwale, among other regions not included in the current study. These ecological zones, together with the Nairobi Metropolitan area, have previously been described by Ogali (2020).

Turkana County on the other hand, is situated on the northwestern part of Kenya. It is the second largest county, covering an area of 77,000 $km^2$ and accounting for 13.5 % of the total land area in the country. The county borders Uganda, South Sudan, and

Ethiopia (Mbuge et al., 2012). The county lies between latitudes 0° 50' and 5° 30' N and longitudes 34° 0' and 36° 40' E. The topography of Turkana county varies between semi-arid and arid landscapes consisting of low-lying plains and isolated hills and mountain ranges. The county has a hot, dry climate with annual mean temperatures experienced in the region ranging between 26°C to 38°C (Mbuge et al., 2012). The rainfall ranges between 120 and 600 mm per year and is bimodal and highly variable, with long rains occurring between April and July and short rains between October and November. The people of Turkana County are traditionally pastoralists, and 60% of the population relies on the livestock sector for their livelihoods (FAO, 2013). Indigenous poultry, especially chicken, is also reared, albeit in low numbers.

### 3.2.3 Study Design and Sample Size Determination

A stratified cross-sectional purposive approach was used during sample collection. The study areas were divided into sub-county populations to reduce sample bias. The maximum possible number of households per sub-county population were then considered, and a total of 647 poultry were sampled. Households were selected based on willingness to participate in the study. A distance of 0.5 km between households were maintained to avoid chances of sampling related individuals (FAO, 2011).

The number of cloacal samples were determined using Fisher's formula (Charan & Kantharia, 2013) as indicated below:

$$\mathbf{n = Z^2P\ (1\text{-}P)/\ e^2}$$ where:

n = estimated sample size

Z = critical value for 95% level of significance (1.96)

P = Proportion of population having the characteristic (0.5)

e = Margin of error (0.1)

n = $1.96^2$ x 0.5 (1-0.5)/ $0.1^2$ = 96 per population (Persoons et al., 2011), making a total of 599 poultry from the six regions of Bungoma, Busia, Nairobi, Trans Nzoia, Kilifi, and Kwale.

The number of faecal and caecal samples per population were determined based on published recommendations by Hale et al. (2012) for population genetic studies, making a total of 48 male and 48 female individuals from six regions (Bungoma, Kilifi, Kwale, Laboot, Siaya, and Turkana).

### 3.2.4 Permits of Compliance and Permission

**The study received institutional clearance from the Jomo Kenyatta University of Agriculture and Technology (JKUAT) to conduct animal research. Permits were also sought from the National Commission for Science, Technology and Innovation (NACOSTI) and the Director of Veterinary Services from the State Department of Livestock, Ministry of Agriculture and Livestock Development, Kenya to study farm animals. Permission was also granted by the respective county governments of Busia, Bungoma, Kilifi, Kwale, Nairobi, Trans Nzoia, and Turkana to conduct the research. The research employed a participatory approach, and prior informed consent from the farmers was sought and given before collecting samples. The birds were handled as humanely as possible, with critical care before, during, and after the sample collection.**

### 3.2.5 Sample Collection

This study was carried out from October 2016 to December 2018 across six counties with varying agroecological conditions in Kenya (Figure 3.1). Cloacal swabs (n=599) were used for detecting enteric viral and bacterial pathogens in poultry and also investigate the presence of antimicrobial resistance genes in poultry. Faecal (n=48) and caecal (n=48) samples on the other hand were used to evaluate the microbial profiles of the different poultry species. Additionally, they were also used for determining antimicrobial resistance genes in the affected poultry species. The total number of samples collected per sample type is shown below in Table 3.1.

**Table 3.1: Total Number of Samples Collected Per Sample Type**

| Sample type | Sample size | Number of pools | Type of analysis |
|---|---|---|---|
| **Cloacal swab** | 599 | 17 | Characterization of viral and bacterial pathogens and detection of ARGs. |
| **Caecal content** | 48 | 16 | Evaluation of microbial community profiles and detection of ARGs |
| **Fecal content** | 48 | 16 | Evaluation of microbial community profiles and detection of ARGs |

Cloacal swabs were collected from 599 poultry from selected regions of Kenya (Figure 3.2).

**Figure 3.2: Collection and Archiving of Poultry Cloacal Samples.**

Information on flock condition or performance were also provided (Appendix I). The cloacal samples were stored in 1 ml of viral transport media (VTM) and immediately put in dry ice and liquid nitrogen afterwards, then later stored at the −80˚C freezer. Two mature birds (above 6 months) per flock from different regions with varying agro-climatic conditions were also selected and sacrificed by cervical dislocation to collect intestinal content (48 poultry in total) and stored in liquid nitrogen (-196ºC) for DNA extraction. Sampling of the 48 poultry was done as per the recommendations of Hale et al. (2012). Caecal and faecal contents were collected and pooled to reduce variation between individuals. Samples were scraped aseptically using sterile glass slides. All samples were then immediately stored in the -80°C freezer until further analysis. Cloacal swabs and faecal and caecal contents collected from each flock were used for detection of viral and bacterial pathogens, analysis of gut microbiomes, and detection of antimicrobial resistance genes in the poultry under study.

Cloacal samples collected from poultry (chickens, ducks, guinea fowls, geese, pigeons, and turkeys) were pooled according to the species and region of origin (Figure 3.3).

**Figure 3.3: Pooling of the Poultry Cloacal Swab Samples.**

The cloacal pools were named CN1-CN6 for chickens, DK1-DK4 for ducks, GF1 for guinea fowls, GS1-GS2 for geese, PN1-PN3 for pigeons, and TY1 for turkeys, resulting in 17 cloacal pools (Table 3.2). The nucleic acids of each pooled sample were used to prepare the viral metagenomic libraries.

**Table 3.2: Pools and Sampling Regions for Cloacal Swab Samples**

| Species | Pool | Number of samples | Region |
|---|---|---|---|
| Chicken | CN1 | 54 | Kilifi |
| | CN2 | 12 | Kilifi |
| | CN3 | 39 | Kwale |
| | CN4 | 77 | Kwale |
| | CN5 | 99 | Nairobi, Trans Nzoia |
| | CN6 | 45 | Busia |
| Duck | DK1 | 19 | Kilifi |
| | DK2 | 31 | Kilifi |
| | DK3 | 18 | Kwale |
| | DK4 | 32 | Bungoma, Busia, Trans Nzoia |
| Guinea fowl | GF1 | 30 | Bungoma, Kilifi, Kwale |
| Goose | GS1 | 28 | Kilifi, Kwale |
| | GS2 | 21 | Bungoma, Busia |
| Pigeon | PN1 | 36 | Kilifi |
| | PN2 | 28 | Bungoma |
| | PN3 | 16 | Busia |
| Turkey | TY1 | 14 | Kilifi |
| **Total** | **17** | **599** | |

*CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey*

Faecal and caecal samples collected from poultry (chickens, ducks, pigeons, and guinea fowls) were also pooled according to the poultry type and region of origin (Figure 3.3 and Tables 3.3 and 3.4).

**Table 3.3: Pooled Faecal Samples Collected from Poultry (Chickens, Ducks, Pigeons, and Guinea Fowls)**

| Poultry type samples | Pooled Sample ID | Sex | Region | Number of |
|---|---|---|---|---|
| Chicken | S001 | Female | Siaya | 3 |
| | S003 | Male | Siaya | 3 |
| | S005 | Female | Laboot | 3 |
| | S007 | Male | Laboot | 3 |
| | S009 | Female | Turkana | 3 |
| | S011 | Male | Turkana | 3 |
| | S013 | Female | Kilifi | 3 |
| | S015 | Male | Kilifi | 3 |
| | S017 | Female | Kwale | 3 |
| | S019 | Male | Kwale | 3 |
| Duck | S021 | Female | Bungoma | 3 |
| | S023 | Male | Bungoma | 3 |
| Pigeon | S025 | Female | Bungoma | 3 |
| | S027 | Male | Bungoma | 3 |
| Guinea fowl | S029 | Female | Bungoma | 3 |
| | S031 | Male | Bungoma | 3 |
| Total | 16 | | | 48 |

*Odd numbers in the sample IDs indicate faecal samples*

**Table 3.4: Pooled Caecal Samples Collected from Poultry (Chickens, Ducks, Pigeons, and Guinea Fowls)**

| Poultry type samples | Pooled Sample ID | Sex | Region | Number of |
|---|---|---|---|---|
| Chicken | S002 | Female | Siaya | 3 |
| | S004 | Male | Siaya | 3 |
| | S006 | Female | Laboot | 3 |
| | S008 | Male | Laboot | 3 |
| | S010 | Female | Turkana | 3 |
| | S012 | Male | Turkana | 3 |
| | S014 | Female | Kilifi | 3 |
| | S016 | Male | Kilifi | 3 |
| | S018 | Female | Kwale | 3 |
| | S020 | Male | Kwale | 3 |
| Duck | S022 | Female | Bungoma | 3 |
| | S024 | Male | Bungoma | 3 |
| Pigeon | S026 | Female | Bungoma | 3 |
| | S028 | Male | Bungoma | 3 |
| Guinea fowl | S030 | Female | Bungoma | 3 |
| | S032 | Male | Bungoma | 3 |
| **Total** | **16** | | | **48** |

*Even numbers in the sample IDs indicate caecal samples*

### 3.3 Analysis of Enteric Viral Pathogens in Poultry

The processing and analysis of viral pathogen data from the cloacal samples is shown in Figure 3.4 below.

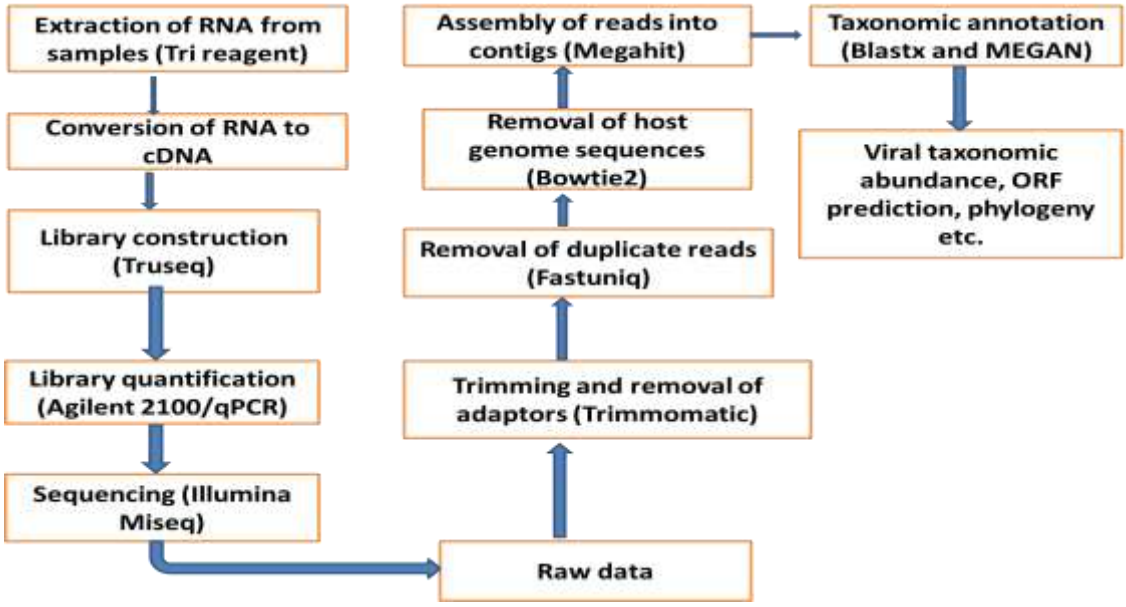


**Figure 3.4: Flow Chart Showing Processing and Analysis of Viral Pathogen Data from the Cloacal Samples.**

#### 3.3.1 RNA Extraction

Viral RNA was extracted from the cloacal swab samples using the standard TRIzol reagent (Rio et al., 2010) according to the manufacturer's instructions with modifications. Briefly, 1 ml Trizol®LS reagent (Invitrogen, California, USA) was added to 200 µl of the cloacal sample and shaken by hand for 20 seconds to mix. The homogenate was incubated for 5 min at room temperature. 200 µl of chloroform was then added, shaken vigorously for 15 seconds, and incubated for 2 to 3 minutes. Thereafter, centrifugation was performed at 12,000 RCF (Relative Centrifugal Force) for 15 minutes at 4ºC. The uppermost layer was then pipetted out into a fresh RNase-free tube to which 500 µl of isopropanol was added, mixed, and incubated for 10 min at room temperature. Centrifugation was then done at 12,000 RCF for 10 minutes at 4ºC. The supernatant was discarded and the pellet washed using 1ml of 75% ethanol. After centrifugation for 5 minutes at 7,500 RCF, the pellet was air-

dried for 5 to 10 minutes, and then resuspended in RNase-free water. Total RNA was then quantified and its integrity checked using the Qubit 4 fluorometer (Invitrogen). The RNA samples were then sent to the International Livestock Research Institute (ILRI) genomic platform where library preparation and whole genome shotgun sequencing were done for cDNA synthesis and sequencing using the Illumina platform.

### 3.3.2 Sequencing of Viral RNA

Viral RNA was reverse-transcribed into complementary DNA (cDNA) using random hexamers in a single step process (Illumina TRUSeq Stranded total RNA Kit, Illumina, Inc, USA). The resulting first strand cDNA was used as template to synthesise the second strand, generating double stranded cDNA (dscDNA) using the same kit. The dscDNA preparation was used as a template to prepare Illumina sequencing library following Illumina DNA prep kit protocol (Illumina, Inc, USA). Indexed multiplexed samples were pooled and reconstituted to 4 nM before diluting to 12 pM for loading into the MiSeq instrument (Illumina, CA, USA) for a 2 x 200 paired-end sequencing run at the ILRI Genomic platform, Nairobi, Kenya. The number of reads obtained from each library are shown in Table 4.1.

### 3.3.3 Bioinformatics Analysis

Poor quality sequencing reads with a Phred quality score < 20 and adaptors were trimmed using Trimmomatic version 0.39 (Bolger et al., 2014). The paired-end sequence reads were *de novo* assembled into contigs using Megahit version 1.0.2 (D. Li et al., 2015a). The assembled contigs were analyzed by BLASTx against a viral protein database and visualized using Megan version 5.5.3 (Huson et al., 2016). Sequences with the best BLAST scores ($E \leq 10^{-3}$) were selected and assigned into known viral families. Overall taxonomic similarities between metagenomes was examined by performing hierarchical clustering and heatmap analyses using the ggplot2 package in RStudio version 3.5.3.

For phylogenetic analyses, sequences representative of known viral families were obtained from GenBank and aligned with the sequences identified in the present study using MUSCLE software (Edgar, 2004). These were used to generate maximum-likelihood phylogenetic trees using PhyML (Guindon et al., 2010) with best fit substitution models determined by Smart Model Selection (Lefort et al., 2017). Statistical significance analyses of tree topologies were performed with the approximate likelihood branch support test (aLRT) or the bootstrap method using 100 replicates (Guindon & Gascuel, 2003).

### 3.4 Analysis of Enteric Bacterial Pathogens

The processing and analysis of enteric bacterial pathogen data from the cloacal samples is shown below (Figure 3.5).

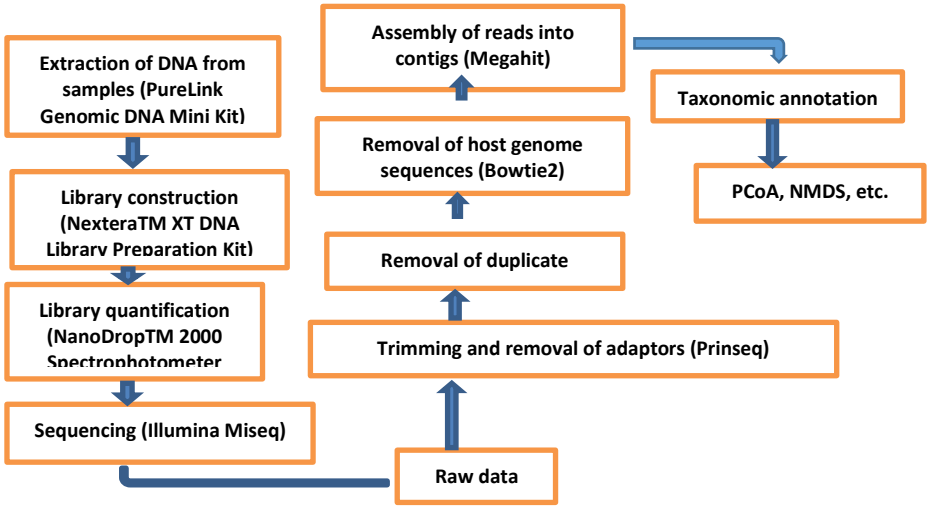


**Figure 3.5: Flow Chart Showing Processing and Analysis of Bacterial Pathogen Data from the Cloacal Samples.**

#### 3.4.1 DNA Extraction

DNA was extracted from the pooled cloacal swabs using the PureLink Genomic DNA Mini Kit (Invitrogen, Life Technologies) following the manufacturer's protocol with modifications. Briefly, the swab was placed into a 2-ml Eppendorf tube to which 200 µl of phosphate-buffered saline (PBS) and 20 µl of Proteinase K were

added and mixed well by pipetting. An equal volume (200 µl) of PureLinkR Genomic Lysis/Binding Buffer was added to the lysate and mixed well by vortexing briefly before incubating at 55°C for at least 10 minutes. The lysate was briefly centrifuged at 10,000 x g and 200 µl of 99 % ethanol added and mixed well by vortexing for 5 seconds. The lysate was then added to a PureLinkR Spin Column attached to a collection tube and centrifuged at 10,000 × g for 1 minute at room temperature. The Collection tube was discarded and the spin column placed into a clean PureLinkR Collection Tube. To wash the extracted DNA, 500 µl of the Wash Buffer 1 prepared with ethanol was added to the column and centrifuged at room temperature at 10,000 × g for 1 minute. The collection tube was discarded and the spin column placed into a clean PureLinkR collection tube. A second washing was done by adding 500 µl of Wash Buffer 2 to the column and centrifuged at maximum speed for 3 minutes at room temperature and the collection tube was discarded. The spin column was finally placed in a sterile 1.5-ml micro-centrifuge tube, and 50 µl of PureLinkR Genomic Elution Buffer was added to the column, which was incubated at room temperature for 1 minute and centrifuged at maximum speed for 1 minute at room temperature. To recover more DNA, a second elution step using the same elution buffer volume as the first was performed in another sterile, 1.5-ml micro-centrifuge tube. The column was then removed and discarded. The purified DNA solution was stored at -20°C freezer before library preparation and sequencing.

### 3.4.2 Library Preparation and DNA Sequencing

The quality and quantity of the DNA preparations were determined in a NanoDropTM 2000 Spectrophotometer and Qubit fluorometer (Invitrogen, ThermoFisher Scientific, Inc., Waltham, Massachusetts, USA), respectively. The extracted genomic DNA was used to prepare dual-indexed paired-end libraries using NexteraTM XT DNA Library Preparation Kit according to the manufacturer's instructions (Illumina, Inc., USA). Indexed samples were pooled and reconstituted to 4 nM before diluting to 12 pM for loading into the MiSeq instrument (Illumina, CA, USA) version 2 reagent kit (300 cycles) with a paired end format (2 x 150 cycles).

### 3.4.3 Taxonomic Assignment

The metagenomic analysis was done using the SqueezeMeta pipeline version 1.5.1 (Tamames &Puente-Sánchez, 2018). Poor quality sequencing reads (short contigs < 200 bp) and adaptors were trimmed using Prinseq version 0.39 (Schmieder & Edwards, 2011). Read mapping against host references was performed to remove host DNA using Bowtie2 version 2.4.5 (Langmead & Salzberg, 2012). The paired-end sequence reads were *de novo* assembled into contigs using Megahit version 1.0.2 (D. Li et al., 2015b). The assembled contigs were used for taxonomic assignment and functional annotation analyses. Taxonomical abundance was determined by comparing metagenomic reads to a database of taxonomically informative gene families to annotate each metagenomic homolog. The whole genome shotgun sequences were therefore taxonomically classified using the RDP classifier (Wang et al., 2007) into taxonomically informative classes. Binning was done using MaxBin2 (Wu et al., 2016) and Metabat2 (Kang et al., 2019). Combination of binning results was done using DAS Tool (Sieber et al., 2018) while bin statistics were computed using CheckM (Parks et al., 2015). The abundance table of different taxonomic ranks was generated based on gene abundance information.

### 3.5 Analysis of Poultry Gut Microbiome

The processing and analysis of poultry gut microbiome data from the faecal and caecal samples is shown in Figure 3.6 below.

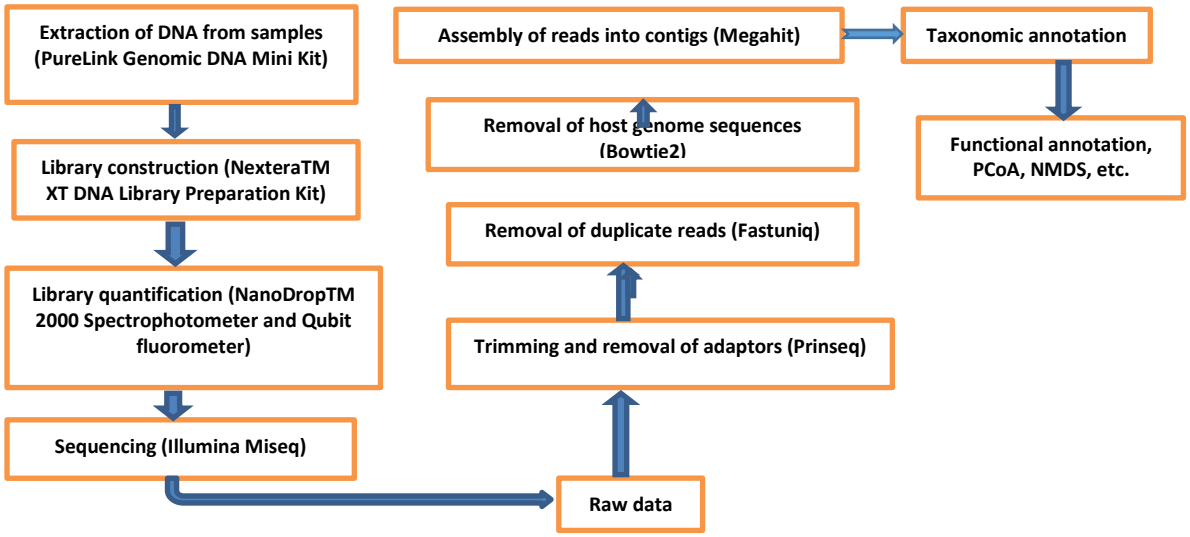


**Figure 3.6: Flow Chart Showing Processing and Analysis of Poultry Gut Microbiome Data from the Faecal and Caecal Samples.**

#### 3.5.1 DNA Extraction

As previously explained, DNA was extracted from the pooled faecal and caecal content using the PureLink Genomic DNA Mini Kit (Invitrogen, Life Technologies) following the manufacturer's protocol with modifications. Briefly, the faecal and caecal contents were collected into a 2-ml Eppendorf tube to which 200 µl of PBS and 20 µl of Proteinase K were added and mixed well by pipetting. An equal volume of PureLinkR Genomic Lysis/Binding Buffer was added to the lysate and mixed well by vortexing briefly before incubating at 55°C for at least 10 minutes. The lysate was briefly centrifuged and 200 µl of 99 % ethanol added and mixed well by vortexing for 5 seconds. The lysate was then added to a PureLinkR Spin Column attached to a collection tube and centrifuged at 10,000 × g for 1 minute at room temperature. The Collection tube was discarded and the spin column placed into a clean PureLinkR Collection Tube. To wash the extracted DNA, 500 µl of the Wash Buffer 1 prepared

with ethanol was added to the column and centrifuged at room temperature at 10,000 × g for 1 minute. The collection tube was discarded and the spin column placed into a clean PureLinkR collection tube. A second washing was done by adding 500 µl of Wash Buffer 2 to the column, and centrifuged at maximum speed for 3 minutes at room temperature before discarding the collection tube. The spin column was finally placed in a sterile 1.5-ml micro-centrifuge tube, and 50 µl of PureLinkR Genomic Elution Buffer was added to the column, which was incubated at room temperature for 1 minute and centrifuged at maximum speed for 1 minute at room temperature. To recover more DNA, a second elution step using the same elution buffer volume as the first was performed in another sterile, 1.5-ml micro-centrifuge tube. The column was then removed and discarded. The purified DNA solution was stored at -20°C freezer until library preparation and sequencing.

### 3.5.2 Sequencing

The quality and quantity of the DNA preparations were determined in a NanoDropTM 2000 Spectrophotometer and Qubit fluorometer (Invitrogen, ThermoFisher Scientific, Inc., Waltham, Massachusetts, USA), respectively. The extracted genomic DNA was used to prepare dual-indexed paired-end libraries using NexteraTM XT DNA Library Preparation Kit according to the manufacturer's instructions (Illumina, Inc., USA). Indexed samples were pooled and reconstituted to 4 nM before diluting to 12 pM for loading into the MiSeq instrument (Illumina, CA, USA) version 2 reagent kit (300 cycles) with a paired end format (2 x 150 cycles) at The Africa Genomics Centre and Consultancy (TAGCC), Nairobi, Kenya.

### 3.5.3 Taxonomic Assignment

The metagenomic analysis was done using the SqueezeMeta pipeline version 1.5.1 (Tamames and Puente-Sánchez, 2018). Poor quality sequencing reads (short contigs < 200 bp) and adaptors were trimmed using Prinseq version 0.39 (Schmieder and Edwards, 2011). Read mapping against host references was performed to remove host DNA using Bowtie2 version 2.4.5 (Langmead and Salzberg, 2012). The paired-end sequence reads were *de novo* assembled into contigs using Megahit version 1.0.2 (D. Li et al., 2015b). The assembled contigs were used for taxonomic assignment and

functional annotation analyses. Taxonomical abundance was determined by comparing metagenomic reads to a database of taxonomically informative gene families to annotate each metagenomic homolog. The whole genome shotgun sequences were therefore taxonomically classified using the RDP classifier (Q. Wang et al., 2007) into taxonomically informative classes. Binning was done using MaxBin2 (Wu et al., 2016) and Metabat2 (D. D. Kang et al., 2019). Combination of binning results was done using DAS Tool (Sieber et al., 2018) while bin statistics were computed using CheckM (Parks et al., 2015). The abundance table of different taxonomic ranks was generated based on gene abundance information.

### 3.5.4 Functional Annotation

The function of the coding sequence was inferred based on similarity to sequences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) as proposed by Kanehisa and Goto (2000) and Clusters of Orthologous Genes (COG) databases using Diamond implementation of the basic alignment search tool (BLAST) (Buchfink et al., 2015) with a cutoff of above 40% of the reference and query ratio being used. The output was a profile describing the number of distinct types of functions and their relative abundance in the metagenome. Clustering, Principal Component Analysis (PCA) and Non-Metric Multi-Dimensional Scaling (NMDS) analyses were performed using the generated taxonomic and functional abundance tables.

### 3.6 Analysis of Antimicrobial Resistance in Poultry

The processing and analysis of antimicrobial resistance data from the cloacal, faecal, and caecal samples is shown in Figure 3.7 below.

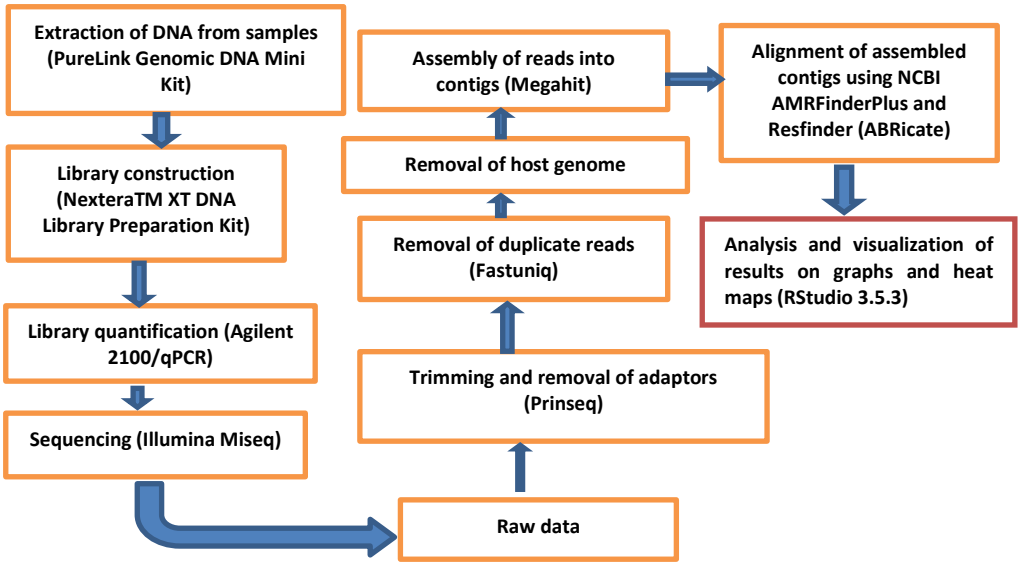


**Figure 3.7: Flow Chart Showing Processing and Analysis of Antimicrobial Resistance Data from the Cloacal, Faecal, and Caecal Samples.**

#### 3.6.1 DNA Extraction

DNA was extracted from the pooled cloacal, faecal, and caecal samples using the PureLink Genomic DNA Mini Kit (Invitrogen, Life Technologies) following the manufacturer's protocol with modifications. Briefly, the sample was placed into 2-ml Eppendorf tube to which 200 µl of PBS and 20 µl of Proteinase K were added and mixed well by pipetting. An equal volume of PureLinkR Genomic Lysis/Binding Buffer was added to the lysate and mixed well by vortexing briefly before incubating at 55°C for at least 10 minutes. The lysate was briefly centrifuged and 200 µl of 99 % ethanol added and mixed well by vortexing for 5 seconds. The lysate was then added to a PureLinkR Spin Column attached to a collection tube and centrifuged at 10,000 × g for 1 minute at room temperature. The Collection tube was discarded and the spin column placed into a clean PureLinkR Collection Tube. To wash the extracted DNA, 500 µl of the Wash Buffer 1 prepared with ethanol was added to the

column and centrifuged at room temperature at 10,000 × g for 1 minute. The collection tube was discarded and the spin column placed into a clean PureLinkR collection tube. A second washing was done by adding 500 µl of Wash Buffer 2 to the column, and centrifuged at maximum speed for 3 minutes at room temperature and the collection tube was discarded. The spin column was finally placed in a sterile 1.5-ml micro-centrifuge tube, and 50 µl of PureLinkR Genomic Elution Buffer was added to the column, which was incubated at room temperature for 1 minute and centrifuged at maximum speed for 1 minute at room temperature. To recover more DNA, a second elution step using the same elution buffer volume as the first was performed in another sterile, 1.5-ml micro-centrifuge tube. The column was then removed and discarded. The purified DNA solution was stored at -20°C freezer until it was processed at The Africa Genomics Centre and Consultancy (TAGCC) for library preparation and whole genome shotgun sequencing.

### 3.6.2 Sequencing

The quality and quantity of the DNA preparations were determined in NanoDropTM 2000 Spectrophotometer and Qubit fluorometer (Invitrogen, ThermoFisher Scientific, Inc., Waltham, Massachusetts, USA), respectively. The extracted genomic DNA was used to prepare dual-indexed paired-end libraries using NexteraTM XT DNA Library Preparation Kit according to the manufacturer's instructions (Illumina, Inc., USA). Indexed samples were pooled and reconstituted to 4 nM before diluting to 12 pM for loading into the MiSeq instrument (Illumina, CA, USA) version 2 reagent kit (300 cycles) with a paired end format (2 x 150 cycles) at The Africa Genomics Centre and Consultancy (TAGCC), Nairobi, Kenya.

### 3.6.3 Characterization of Antimicrobial Resistance Genes (ARG)

#### 3.6.3.1 Characterization of Antimicrobial Resistance Genes in Poultry Cloacal Samples

Antimicrobial resistance genes (ARGs) from the poultry cloacal swab content were characterized to explore the relationship between diverse sequences and resistance levels. The assembled contigs of cloacal swabs of the different poultry species were

aligned against the NCBI AMRFinderPlus (Feldgarden et al., 2019) and Resfinder (Zankari et al., 2012) databases for mass screening of the assembled contigs for ARGs using ABRicate software version 1.0.1 (Seemann, 2014/2024) (https://github.com/tseemann/abricate).

Based on raw read counts, the relative abundances of AMR genes were estimated. Analysis and visualization of results on graphs and heat maps were carried out in the open source RStudio version 3.5.3 for Windows (https://www.rproject.org/) using the library (vegan), library(ggplot2), library(reshape2), and library(RColorBrewer) packages. The ARGs' relative abundance in the cloacal swabs and their distribution through hierarchical clustering in all classification levels are reported.

#### 3.6.3.2 Characterization of Antimicrobial Resistance Genes in Poultry Faeces and Caeca

The antimicrobial resistance genes (ARGs) from the poultry faecal and caecal content were characterized to explore the relationship between diverse sequences and resistance levels. The assembled contigs of faecal and caecal samples of the different poultry species were aligned against the NCBI AMRFinderPlus (Feldgarden et al., 2019) and Resfinder (Zankari et al., 2012) databases for mass screening of the assembled contigs for ARGs using ABRicate software version 1.0.1 (Seemann, 2014/2024) (https://github.com/tseemann/abricate).

Based on raw read counts, the relative abundances of AMR genes were estimated. Analysis and visualization of results on graphs and heat maps were carried out in the open source RStudio version 3.5.3 for Windows (https://www.rproject.org/) using the library (vegan), library(ggplot2), library(reshape2), and library(RColorBrewer) packages. The ARG's relative abundance between the faecal and caecal samples and their distribution through hierarchical clustering in all classification levels are reported.

# CHAPTER FOUR

# RESULTS

## 4.1 Introduction

This chapter presents the results of bioinfomatic and statistical analyses carried out as per each specific objective.

## 4.2 Characterization of Enteric Viral Pathogens

### 4.2.1 General Overview of the Viral Pathogen Sequence Data

A total of 26,044,021 raw reads and 17,034,948 clean paired-end reads with an average of 200 nt, were generated from the poultry cloacal swab samples (Table 4.1). From the clean paired-end reads generated, 9,131 contigs were assembled. Using BLASTx and BLASTn analyses, a total of 177 *de novo* assembled contigs were identified with hits to known viral sequences. The distribution of viral sequences and their detection rate in the pooled poultry cloacal samples are shown in Table 4.1 below.

**Table 4.1: Number of Raw Reads, Clean Reads, Assembled Contigs, Viral Contigs, and Viruses Identified Per Cloacal Swab Sample**

| Sample | Number of raw reads | Number of clean reads | Number of assembled contigs | Number of viral contigs | Number of identified viral sequences |
|---|---|---|---|---|---|
| CN1 | 325,059 | 216,990 | 104 | 12 | 10 |
| CN2 | 1,143,251 | 848,599 | 183 | 1 | 0 |
| CN3 | 478,941 | 403,451 | 262 | 2 | 2 |
| CN4 | 725,247 | 610,624 | 350 | 81 | 70 |
| CN5 | 1,427,756 | 1,159,753 | 463 | 74 | 62 |
| CN6 | 934,445 | 632,697 | 184 | 0 | 0 |
| DK1 | 2,584,056 | 2,211,786 | 839 | 11 | 11 |
| DK2 | 1,146,938 | 965,013 | 778 | 2 | 0 |
| DK3 | 6,714,045 | 5,336,610 | 3,195 | 22 | 13 |
| DK4 | 1,790,893 | 1,451,922 | 606 | 7 | 2 |
| GF1 | 1,192,516 | 541,072 | 312 | 2 | 0 |
| GS1 | 1,121,473 | 476,373 | 331 | 6 | 0 |
| GF2 | 1,165,512 | 610,108 | 368 | 3 | 1 |
| PN1 | 1,699,746 | 582,510 | 390 | 24 | 1 |
| PN2 | 1,416,584 | 481,074 | 274 | 1 | 0 |
| PN3 | 604,651 | 54,859 | 24 | 0 | 0 |
| TY1 | 1,572,908 | 451,507 | 468 | 6 | 5 |
| **Total** | **26,044,021** | **17,034,948** | **9,131** | **254** | **177** |

*CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey*

### 4.2.2 Viral Abundances

The family level viral abundances in each pooled sample are shown below in a piechart (Figure 4.1). Most of the detected potentially pathogenic viruses belonged to the *Coronaviridae* (43.4%) and *Reoviridae* (36.6%) families. Other potentially pathogenic viruses detected in smaller proportions belonged to *Retroviridae* (11.4%), the unclassified Deltavirus (1.7%), and *Picobirnaviridae* (0.6%).

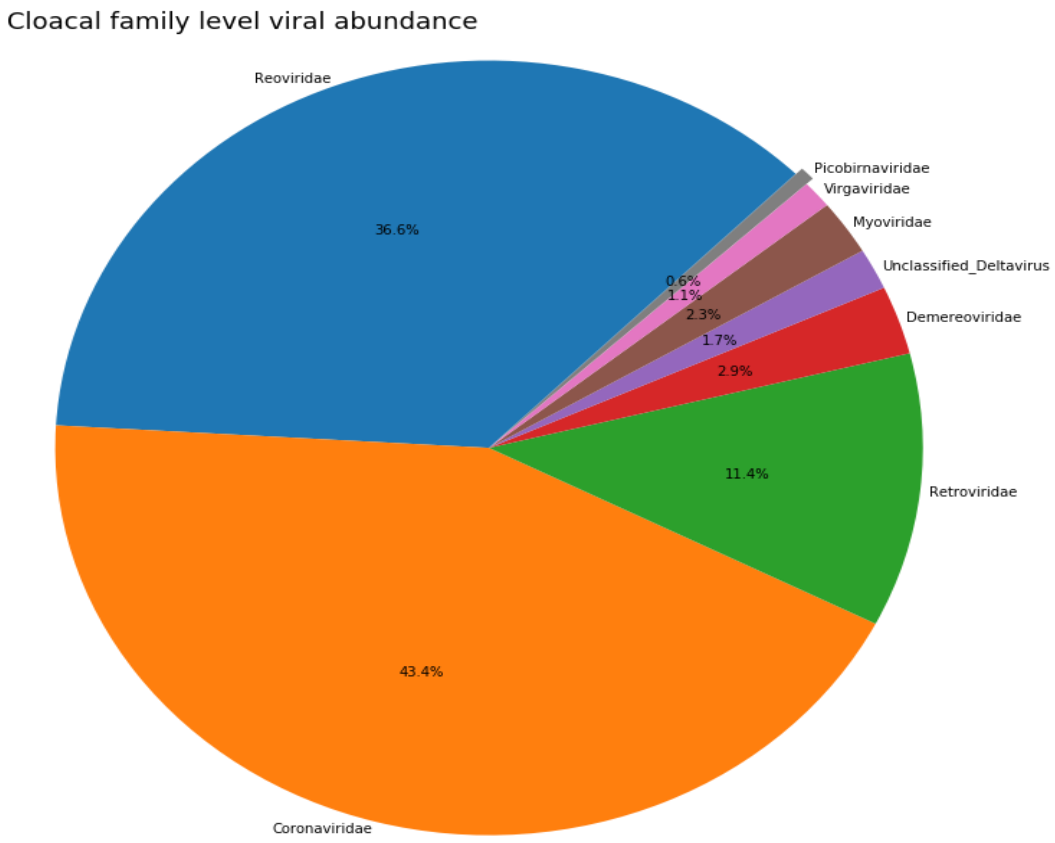


**Figure 4.1: Family Level Viral Abundances. The Pie Chart was Plotted Using Seaborn in Python Version 3.7.**

The species level viral abundances in each pooled sample are shown below in a piechart (Figure 4.2). The most abundant viral species detected were avian infectious bronchitis virus (IBV) (42.6%) and rotavirus (35.0%). Other potentially pathogenic viruses detected were reticuloendotheliosis virus (REV) (6.0%), lymphoproliferative disease virus (LDV) (2.7%), turkey coronavirus (TCoV) (1.6), avian leucosis virus (ALV) (1.6), avian HDV-like agent (1.6%), avian coronavirus (1.1%), pigeon-dominant coronavirus (0.5%), and picobirnavirus strain HK-2014 (0.5%).

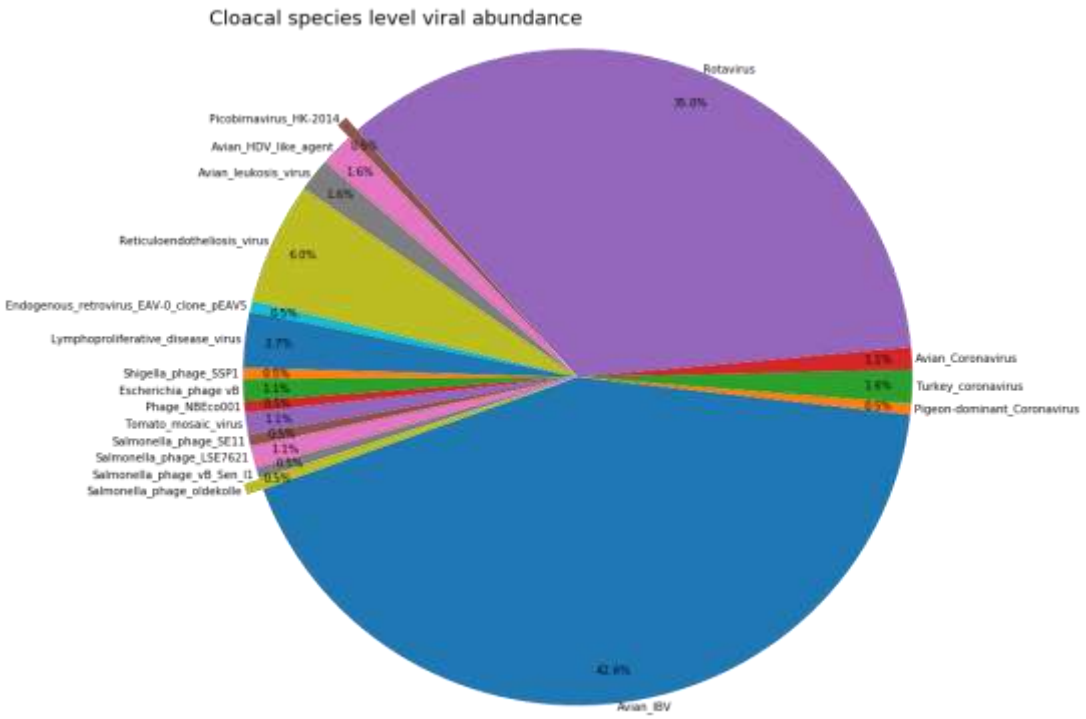


**Figure 4.2: Species-Level Viral Abundances. The Pie Chart was Plotted Using Seaborn in Python Version 3.7.**

The relative abundance of poultry viruses at family level is shown below in a bargraph Figure 4.3. The most frequently detected viral families were *Reoviridae* (37.14-100%), *Coronaviridae* (50-62.86%) and *Retroviridae* (1.61-100%). The most abundant viral families in ducks were *Retroviridae* (9.09-90.91%), unclassified Deltavirus (9.09-50%) and *Picobirnaviridae* (9.09%). *Coronaviridae* and *Retroviridae* were also detected in one pigeon and turkey sample each, respectively.

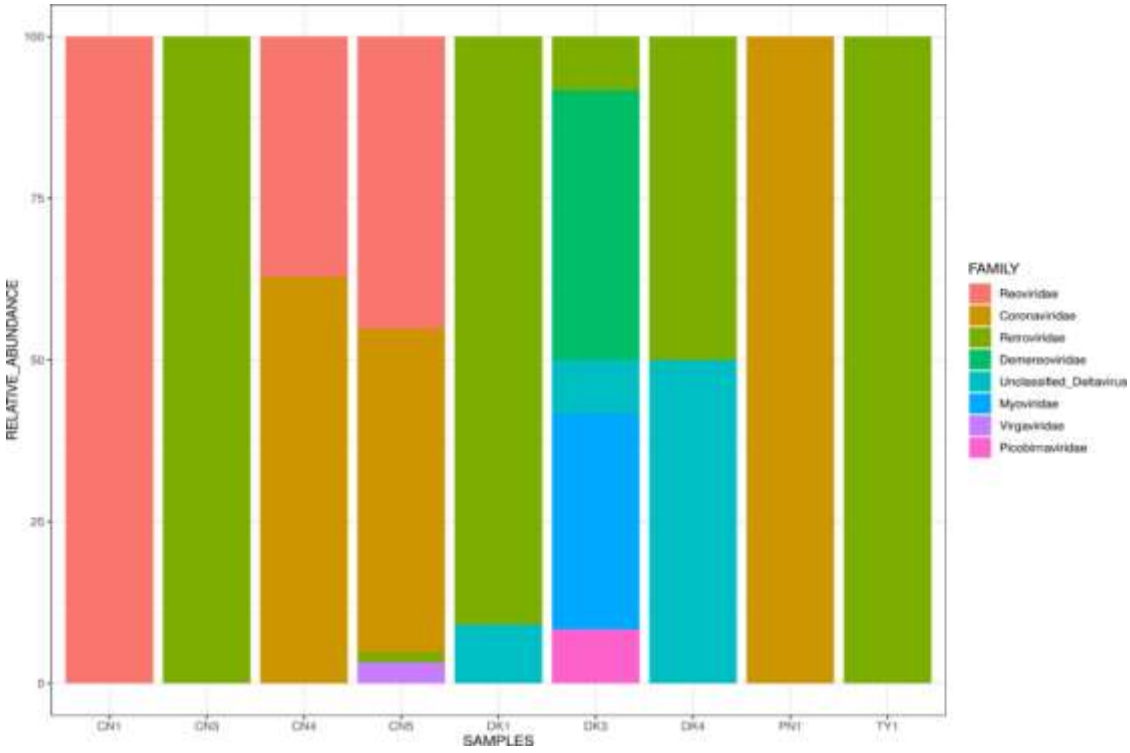


**Figure 4.3: Classification of Viral Contigs Detected from Poultry Plotted Using Ggplot2 in R Studio Version 4.0.3.** A stacked column chart with taxonomic relative abundances (y-axis) by samples (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. Only samples with detectable viruses are shown. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

The relative abundance of poultry viruses at species level is shown below in a bargraph (Figure 4.4). The most frequently detected viral species in chicken were rotavirus (37.14-100%) and avian IBV (45.16-60%). Other coronaviruses such as TCoV and Avian coronavirus were also detected at 1.49-3.23% and 1.43-1.61% respectively. ALV was likewise detected in one chicken sample. The most frequently detected viral species in ducks were REV (50-90.91%) and avian HDV-like agent (8.33-50%). Other viruses detected in ducks were ALV (8.33%), Picobirnavirus (6.33%) and Endogeneous retrovirus strain EAV-0 (1.61%). LDV and Pigeon-dominant coronavirus were also detected in turkeys and pigeons, respectively.

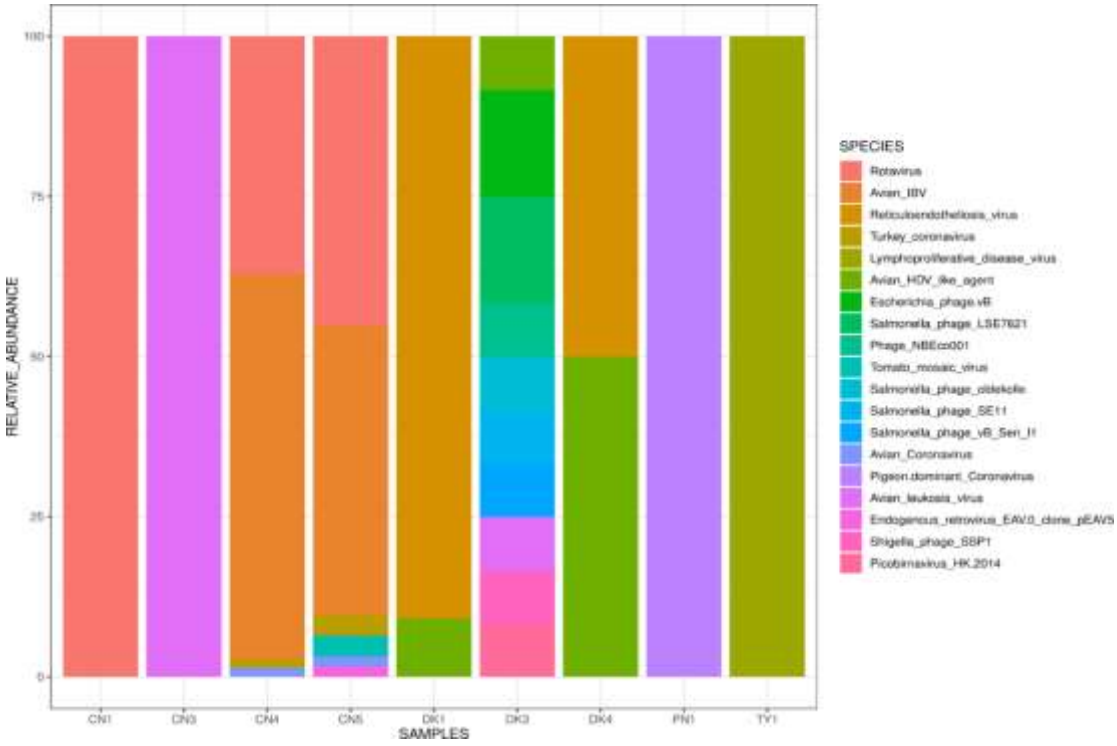


**Figure 4.4: Bar Graph Showing Species-Level Relative Viral Abundance in Each Sample Plotted Using Ggplot2 in R Studio Version 4.3.0.** A stacked column chart with taxonomic relative abundances (y-axis) by sample (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. Only samples with detectable viruses are shown. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

In order to assess the relatedness and overall taxonomic similarities between the identified sequences in the studied poultry, a hierarchical clustering analysis was performed, augmented by heatmaps. The clustermaps and heatmaps of species level viral abundances are shown below in Figure 4.5 and 4.6, respectively. Heatmaps generated did not reveal any distinct pattern for the samples from the different poultry. The heatmaps also corroborate the findings that show rotavirus and infectious bronchitis virus to have been detected in higher numbers in some chicken samples. The hierarchical clustermaps on the other hand had dendrograms with intermingled branches, implying no clear separation between samples from the different poultry.

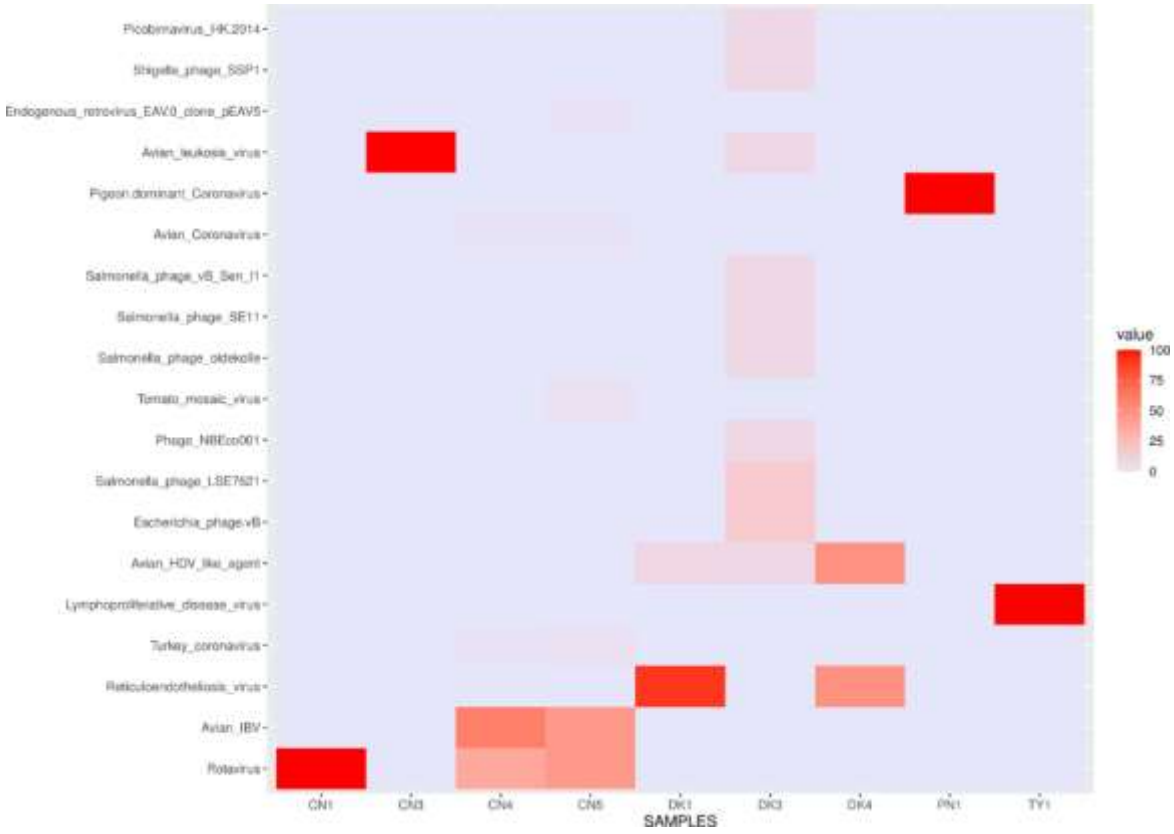


**Figure 4.5: Taxonomic Abundances Heatmap Based on Log-Transformed Relative Abundance Values of Viral Abundance in Each Species (Y-Axis) in all Samples (X-Axis) Plotted Using Ggplot2 in R Version 4.3.0.** Only samples with detectable viruses are shown. Colors scale from red (high abundance) to lavender (low abundance) represent log-transformed relative abundance. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

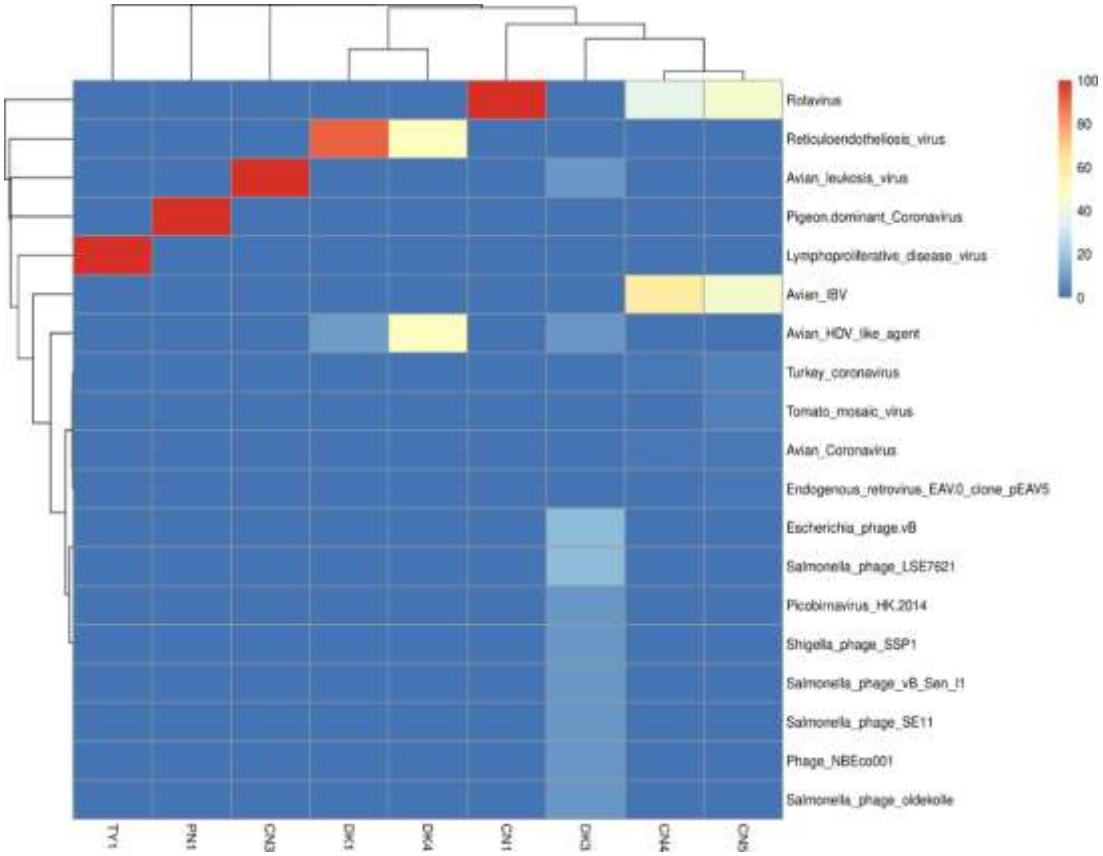


**Figure 4.6: Taxonomic Abundances Clustermap Based on Log-Transformed Relative Abundance Values of Viral Abundance in Each Species (Y-Axis) in All Samples (X-Axis) Plotted Using Ggplot2 and Pheatmap, Respectively, in R Version 4.3.0.** Only samples with detectable viruses are shown. Colors scale from red (high abundance) to blue (low abundance) represent log-transformed relative abundance. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

### 4.2.3 Detected Viruses in Poultry

Sequences identified in poultry cloacal samples were assigned to five viral families, including *Coronaviridae*, *Picobirnaviridae*, *Reoviridae*, *Retroviridae*, and the unclassified Deltavirus.

#### 4.2.3.1 Coronaviridae

Seventy-six coronavirus-related contigs (75 from chicken and 1 from pigeon) were recovered from the poultry samples (Table 4.2). These contigs ranged from 316 to 5,516 nt in length and displayed between 83.46 to 100% amino acid identity to other coronavirus sequences deposited in the NCBI Genbank database (data not shown). Majority of the coronavirus sequences belonged to avian IBV (70 contigs). Turkey coronavirus, avian coronavirus, and Pigeon-dominant coronavirus were also detected, albeit in lower numbers. Most of the coronavirus sequences were detected in chicken samples collected from Kwale, Nairobi, and Trans Nzoia (detection rate 2/6 or 33.33%), with only 1 sequence being detected in a pigeon sample collected from Kilifi (Table 4.3). Contigs showing identity to the S protein were selected to construct a phylogenetic tree. The ML phylogenetic analysis supports the classification of the coronavirus sequences reported in this study at the species level (Figure 4.7).

#### 4.2.3.2 Reoviridae

Sixty-four rotavirus-related contigs were recovered from chicken samples (Table 4.2). The rotavirus sequences were detected in chicken samples collected from Kilifi, Kwale, Nairobi, and Trans Nzoia (detection rate 3/6 or 50%) (Table 4.3). Contigs showing identity to the VP6 protein were selected to construct a phylogenetic tree. Phylogenetic analysis of the VP6 protein amino acid sequences confirmed a close relationship between the detected rotavirus sequences and other previously described rotavirus sequences (Figure 4.8).

#### 4.2.3.3 Retroviridae

Twenty retrovirus-related contigs (15 from ducks and 5 from turkeys) were recovered (Table 4.2). Majority of the retroviral sequenced detected mapped to REV (11 sequences), followed by the LDV (5 sequences), and the ALV (3 viral contigs), with 1 contig mapping to the Endogenous retrovirus EAV-0_ lone pEAV5 (Tables 4.2 and 4.3).

Other potentially pathogenic viruses recovered from poultry included Avian HDV-like agent (3 from duck samples) and Picobirnavirus HK-2014 (1 from duck sample). The rest of the viral sequences mapped to the Salmonella phage LSE7621 (2 from duck sample), Salmonella phage SE11 (1 from duck sample), Salmonella phage vB Sen I1 (1 from duck sample), Salmonella phage oldekoll (1 from duck sample), Escherichia phage Vb (2 from duck sample), Shigella phage SSP1 (1 from duck sample), Tomato mosaic virus (2 from chicken sample), and Phage NBEco001 (1 sequence from duck sample).

## Table 4.2: Number of Contigs and Detection Rate of Viruses in Poultry Cloacal Swab Samples

| Detected viruses | Samples CN1 | CN2 PN1 | CN3 PN2 | CN4 PN3 | CN5 TY1 | CN6 | DK1 | DK2 | DK3 | DK4 | GF1 | GS1 | GS2 | Detection rate (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Avian IBV | | | | 42 | 28 | | | | | | | | | 2/6 (33.33) |
| Rotavirus | 10 | | | 26 | 28 | | | | | | | | | 3/6 (50.0) |
| REV | | | | | | | 10 | | | 1 | | | | 2/4 (50.0) |
| Lymphoproliferative disease virus | | | | 5 | | | | | | | | | | 1/1 (100) |
| Turkey coronavirus | | | | | | | | | | | | | | 3/6 (50.0) |
| Avian HDV-like agent | | | | 1 | 2 | | 1 | | 1 | 1 | | | | 3/17 (17.65) |
| Avian leukosis virus | | | | | | | | | | | | | | |
| Avian coronavirus | | | 2 | | | | | | 1 | | | | | 2/17 (11.76) |
| Salmonella phageLSE7621 | | | | 1 | 1 | | | | | | | | | 2/17 11.76) |
| Tomato mosaic virus | | | | | | | | | 2 | | | | | 1/17 (5.88) |
| Escherichia phage Vb | | | | | 2 | | | | 2 | | | 1 | | 1/17 (5.88) |
| Picobirnavirus HK-2014 | | | | | | | | | | | | | 1 | 1/17 (5.88) |
| Pigeon-dominant | | | | | | | | | | | | | | |

| Detected viruses | Samples CN1 | CN2 PN1 | CN3 PN2 | CN4 PN3 | CN5 TY1 | CN6 | DK1 | DK2 | DK3 | DK4 | GF1 | GS1 | GS2 | Detection rate (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| coronavirus | | | | | | | | | | | | | | |
| Salmonella phage SE11 | | | | | | | | | | | | | | 1/17 (5.88) |
| | | | | | | | | | 1 | | | | | |
| Endogenous retrovirus EAV-0_ lone pEAV5 | | | | | | | | | | | | | | 1/17 (5.88) |
| Salmonella phage vB Sen I1 | | | | | | | | | 1 | | | | | |
| | | | | | | | | | | | | | | 1/17 (5.88) |
| Salmonella phage oldekoll | | | | | | | | | 1 | | | | | |
| Shigella phage SSP1 | | | | | | | | | 1 | | | | | 1/17 (5.88) |
| Phage NBEco001 | | | | | | | | | | | | | | 1/17 (5.88) |
| | | | | | | | | | 1 | | | | | |
| | | | | | | | | | | | | | | 1/17 (5.88) |
| | | | | | | | | | 1 | | | | | |
| | | | | | | | | | 1 | | | | | 1/17 (5.88) |
| | | | | | | | | | | | | | | 1/17 (5.88) |

*CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey*

**Table 4.3: Detected Viruses in Cloacal Swabs of Kenyan Poultry**

| Poultry | Virus detected | County | Sample ID | Detection ratespecies |
|---|---|---|---|---|
| Chicken | Rotavirus | Kilifi, Kwale, Nairobi, Trans Nzoia | CN1, CN4, CN5 | 3/6 (50%) |
| | Avian IBV | Kwale, Nairobi, Trans Nzoia | CN4, CN5 | 2/6 (33.33%) |
| | Turkey coronavirus | Kwale, Nairobi, Trans Nzoia | CN4, CN5 | 2/6 (33.33%) |
| | Avian coronavirus | Kwale | CN4 | 1/6 (16.67%) |
| Duck | Avian HDV-like virus | Kilifi, Kwale, Bungoma, Busia, Trans Nzoia | DK1, DK3, DK4 | 3/4 (75%) |
| | REV | Kilifi, Bungoma, Busia, Trans Nzoia | DK1, DK4 | 2/4 (50%) |
| | ALV | Kwale | DK3 | 1/4 (25%) |
| | Picobirnavirus | Kwale | DK3 | 1/4 (25%) |
| Pigeon | Pigeon-dominant coronavirus | Kilifi | PN1 | 1/3 (33.33) |
| Turkey | LDV | Kilifi | TY1 | 1/1 (100%) |

*CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey*

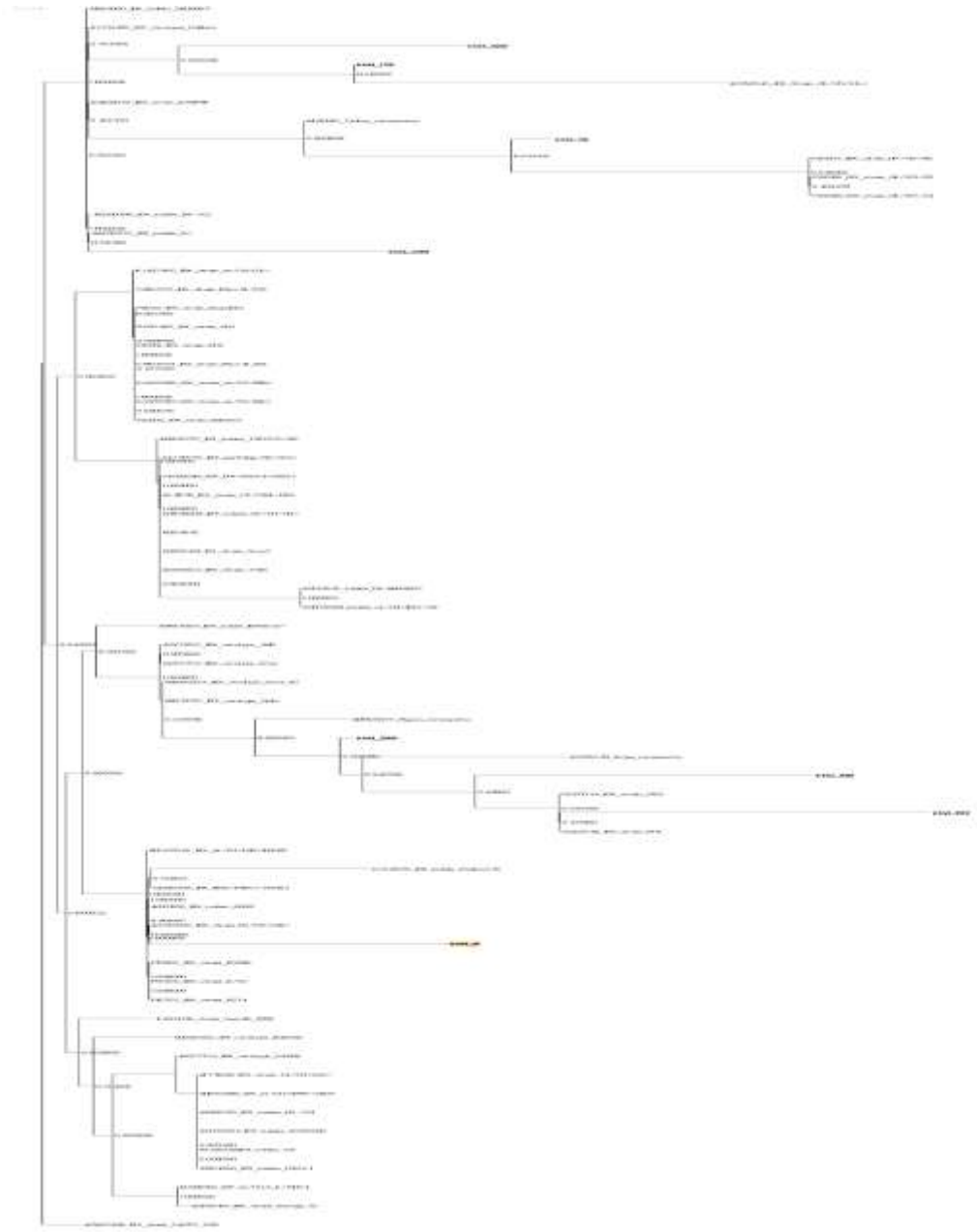

**Figure 4.7: Phylogeny of the Coronavirus Sequences Detected in Poultry Cloacal Samples and Other Sequences Downloaded from Genbank Based on Amino Acid Sequences of the S Protein.** Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap analysis with 100 replicates.

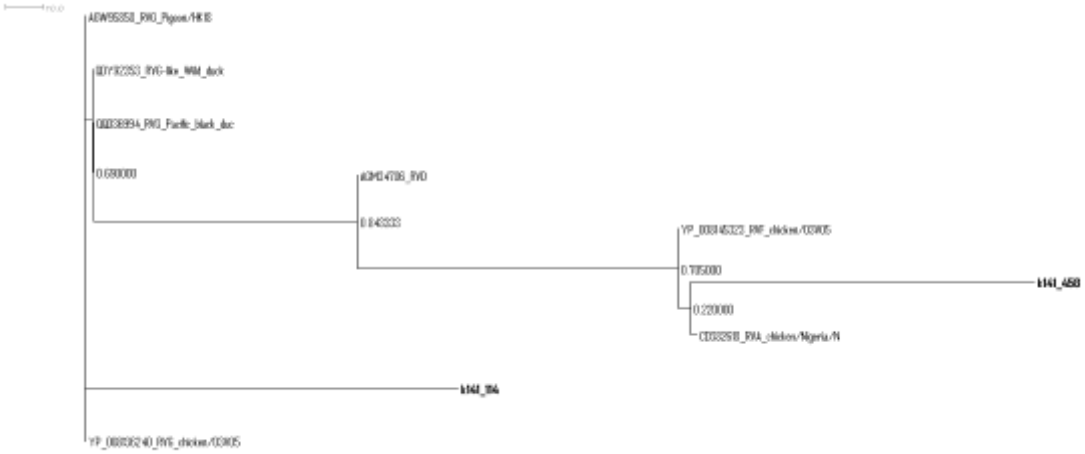

**Figure 4.8: Phylogeny of the Rotavirus Sequences Detected in Poultry Cloacal Samples and other Sequences Downloaded from Genbank Based on Amino Acid Sequences of the S Protein.** Maximum likelihood tree generated using PhyML using the LG amino acid substitution model. Branch support was estimated by bootstrap analysis with 100 replicates.

### 4.3 Characterization of Enteric Bacterial Pathogens

#### 4.3.1 Overview of the Bacterial Pathogen Sequence Data

A total of 17,002,195 paired-end reads with a median length of 200 base pairs (bp) were obtained from all the poultry cloacal samples (Tables 4.4). The total number of clean reads generated from the cloacal samples were 16,432,416. These were subsequently assembled into a total of 66,090 contigs. Using a 95% similarity cut-off, the assembled contigs yielded 315 operational taxonomic units (OTUs). Two samples (CN3 and DK3) were not informative as they did not generate any OTUs that could be used for taxonomic assignment.

Rarefaction (discovery) curves generated from the OTUs show that all the samples approached a plateau, which suggests that the sample volumes were efficient in estimating the detected taxa in the samples (Figure 4.9).

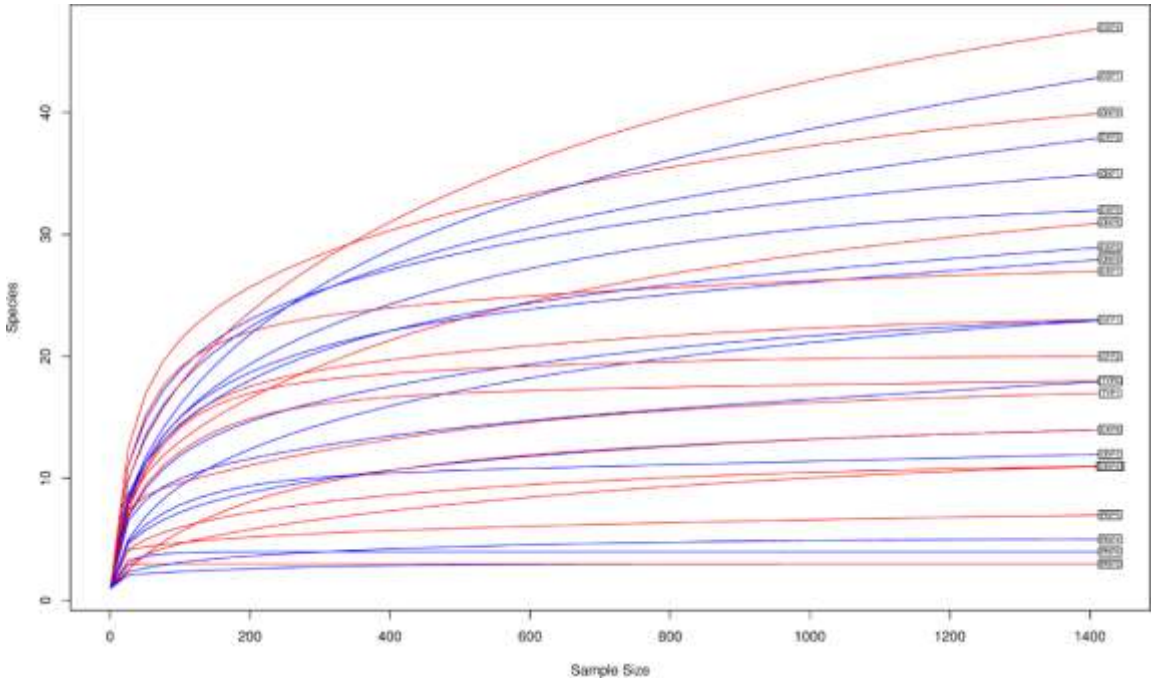


**Figure 4.9: Rarefaction Curves of Poultry Cloacal Samples Clustered at 90% Sequence Identity.** The rarefaction curves for each sample were plotted without replacement. Rarefaction is used to simulate an even number of reads per sample. In this study, the rarefaction depth chosen is 90% of the minimum sample depth in the dataset. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

The species richness (observed number of OTUs and ACE) and community diversity (Chao1, Shannon, and Inverse Simpson indices) are shown in Table 4.5 below. Analysis of these diversity indices showed that there was no significant difference in species richness and diversity across the poultry except for the pigeons, which had much lower richness and diversity compared to other poultry (Table 4.5). This implies that the species richness and diversity of the bacterial pathogens that colonize the cloacal regions are generally similar across the different poultry.

**Table 4.4: Number of Raw Reads, Clean Reads, Assembled Contigs, and Observed Number of OTUS Identified in Cloacal Swab Samples**

| Sample | Number of raw reads | Number of clean reads | Number of assembled contigs | Number of observed OTUs |
|---|---|---|---|---|
| CN1 | 739,235 | 727,578 | 1,210 | 35 |
| CN2 | 721,871 | 709,914 | 6,181 | 12 |
| CN3 | 1,316,673 | 1,294,994 | 5,314 | |
| CN4 | 962,476 | 946,762 | 1,312 | 11 |
| CN5 | 1,183,395 | 1,164,692 | 3,553 | 23 |
| CN6 | 972,083 | 954,980 | 3,704 | 31 |
| DK1 | 1,083,848 | 1,064,236 | 2,268 | 27 |
| DK2 | 1,866,041 | 1,836,634 | 5,244 | 32 |
| DK3 | 1,381,344 | 1,064,236 | 11,450 | |
| DK4 | 1,254,999 | 1,235,172 | 3,004 | 23 |
| GF1 | 1,078,026 | 1,060,498 | 4,361 | 43 |
| GS1 | 1,181,800 | 1,163,704 | 4,220 | 18 |
| GS2 | 1,366,750 | 1,345,650 | 7,775 | 23 |
| PN2 | 243,351 | 239,606 | 406 | 20 |
| PN3 | 183,080 | 179,774 | 236 | 3 |
| TY2 | 1,467,223 | 1,443,986 | 5,852 | 14 |
| **Total** | **17,002,195** | **16,432,416** | **66,090** | **315** |

*CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey*

**Table 4.5: OTUs (0.05% Coverage) and Diversity Indices from Cloacal Samples from Different Poultry**

| Sample | Number of Observed OTUs | Chao1 | ACE | Shannon | Inverse Simpson |
|---|---|---|---|---|---|
| CNP1 | 35 | 38.00 | 40.23 | 2.548 | 8.310 |
| CNP2 | 12 | 13.00 | 16.09 | 1.261 | 2.544 |
| CNP4 | 11 | 11.00 | 11.69 | 1.146 | 2.414 |
| CNP5 | 23 | 25.50 | 27.06 | 1.244 | 2.245 |
| CNP6 | 31 | 35.20 | 35.12 | 1.929 | 4.112 |
| DKP1 | 27 | 27.33 | 29.03 | 2.498 | 7.513 |
| DKP2 | 32 | 32.50 | 33.68 | 2.009 | 4.195 |
| DKP4 | 23 | 23.00 | 23.88 | 1.761 | 2.989 |
| GSP1 | 43 | 58.60 | 55.18 | 2.150 | 5.237 |
| GSP2 | 18 | 18.00 | 18.42 | 1.651 | 2.985 |
| GFP1 | 23 | 23.60 | 25.37 | 1.612 | 2.852 |
| GFP2 | 20 | 20.00 | 20.00 | 1.674 | 2.811 |
| PNP2 | 3 | 3.00 | 3.00 | 0.405 | 1.294 |
| PNP3 | 3 | 3.00 | NaN | 0.913 | 2.198 |
| TYP1 | 17 | 17.50 | 17.75 | 1.939 | 5.419 |
| Total | 301 | | | | |

*CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey*

### 4.3.1 Bacterial Pathogen Composition

The relative abundance at the phylum level for individual samples are shown below in Figure 4.10. *Chlamydiae* (11.38-98.43%) and *Proteobacteria* (1.57-85.46%) were the most frequently detected phyla in the chickens while *Proteobacteria* (28.51-100%) and *Firmicutes* (3.13-100%) were the most frequently detected in other poultry. *Proteobacteria* were especially noted to be frequent in most poultry in the current study.

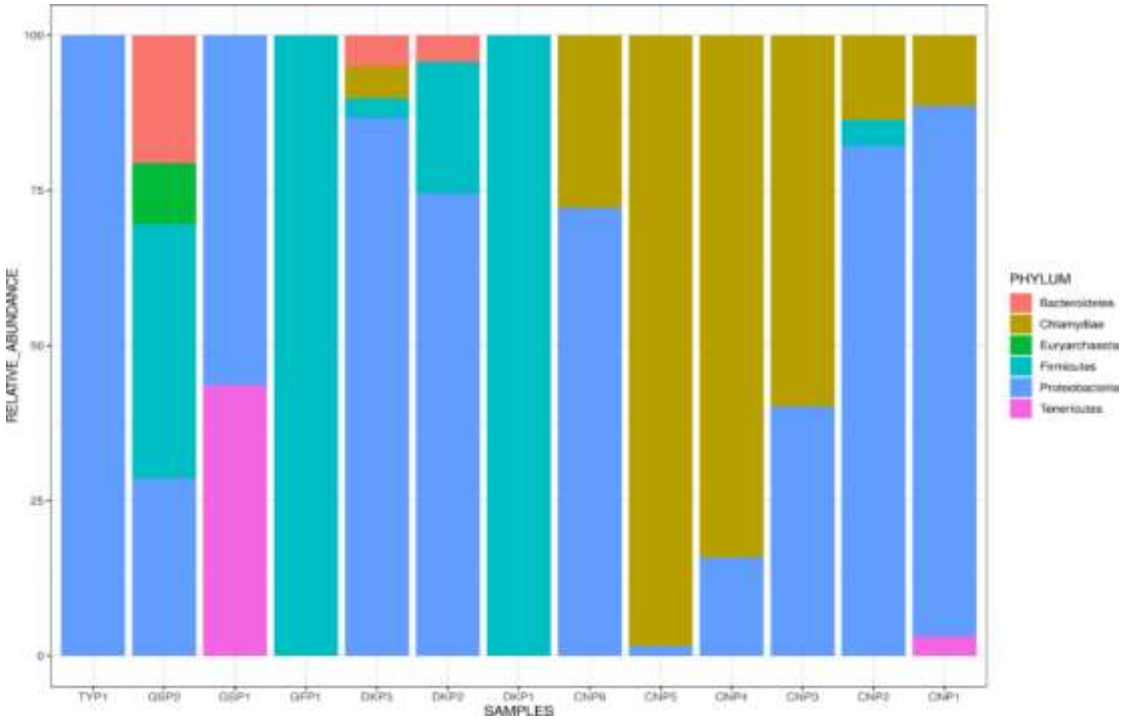


**Figure 4.10: Bacterial Relative Abundance at Phylum Level (Y-Axis) in all Cloacal Samples (X-Axis).** A stacked column chart with taxonomic relative abundances (y-axis) by sample (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

Class-level relative abundance for individual samples are shown below (Figure 4.11). *Gammaproteobacteria* were the most frequently detected across all the poultry while *Chlamydiia* were detected in all the chicken samples and some duck samples. *Bacilli*, *Bacteroidia*, and *Erysipelotrichia* on the other hand, were detected in ducks and geese. Other bacteria detected in smaller quantities were *Mollicutes*, *Negativicutes*, and *Methanobacteria*.

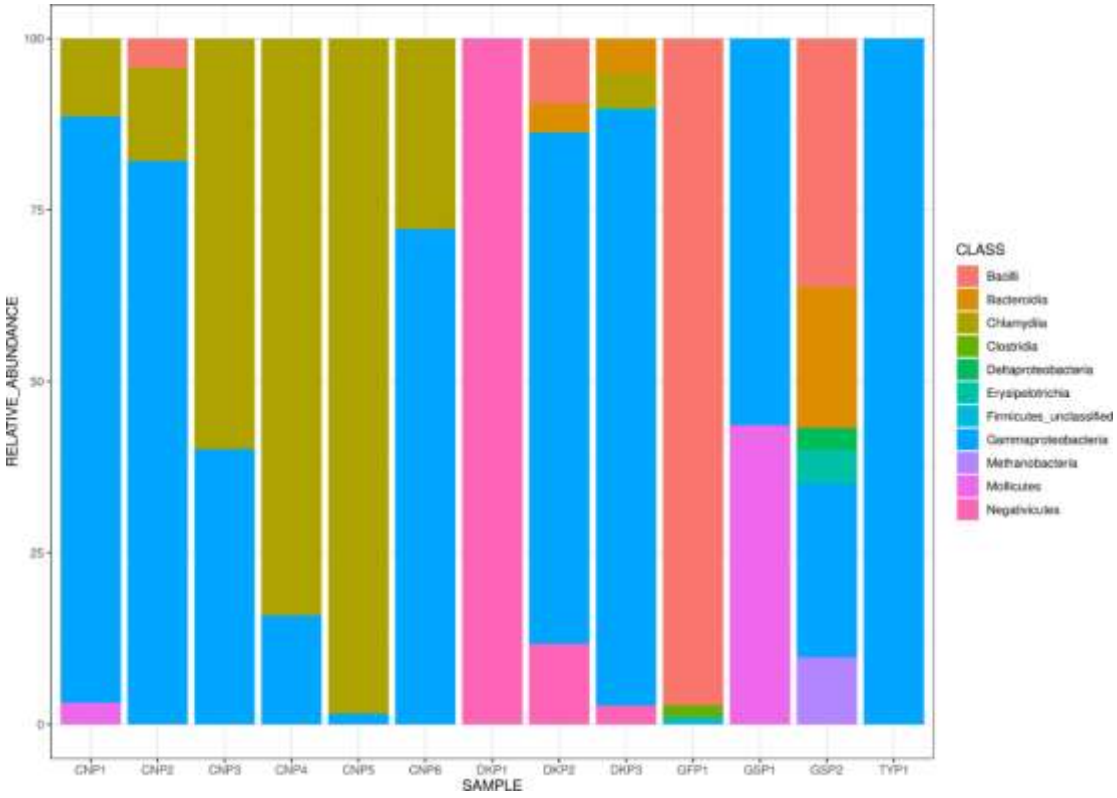


**Figure 4.11: Bacterial Relative Abundance at Class Level (Y-Axis) in all Cloacal Samples (X-Axis).** A stacked column chart with taxonomic relative abundances (y-axis) by sample (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

The relative abundance at the order level are also shown (Figure 4.12). The most frequently detected orders were *Enterobacterales*, *Chlamydiales*, and *Lactobacillales* while *Bacteroidales*, *Selenomonadales*, and *Erysipelotrichales* were only detected in ducks and geese.

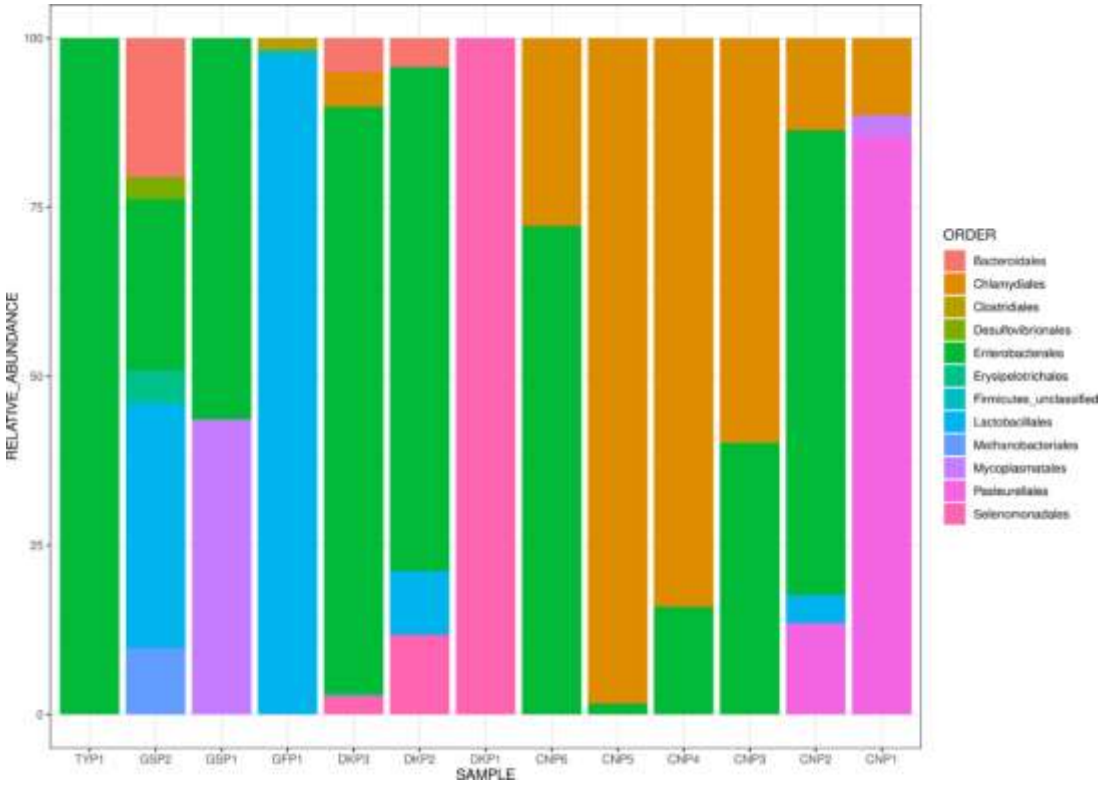


**Figure 4.12: Bacterial Relative Abundance at Order Level (Y-Axis) in all Cloacal Samples (X-Axis).** A stacked column chart with taxonomic relative abundances (y-axis) by sample (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

Family-level distributions for individual samples are shown below (Figure 4.13). The most dominant bacteria were *Enterobacteriaceae* and *Chlamydiaceae* while *Pasteurellaceae* were only detected in chickens. On the other hand, *Enterococcaceae* were detected in chickens, ducks and geese while *Mycoplasmataceae* were found only in chickens and geese.

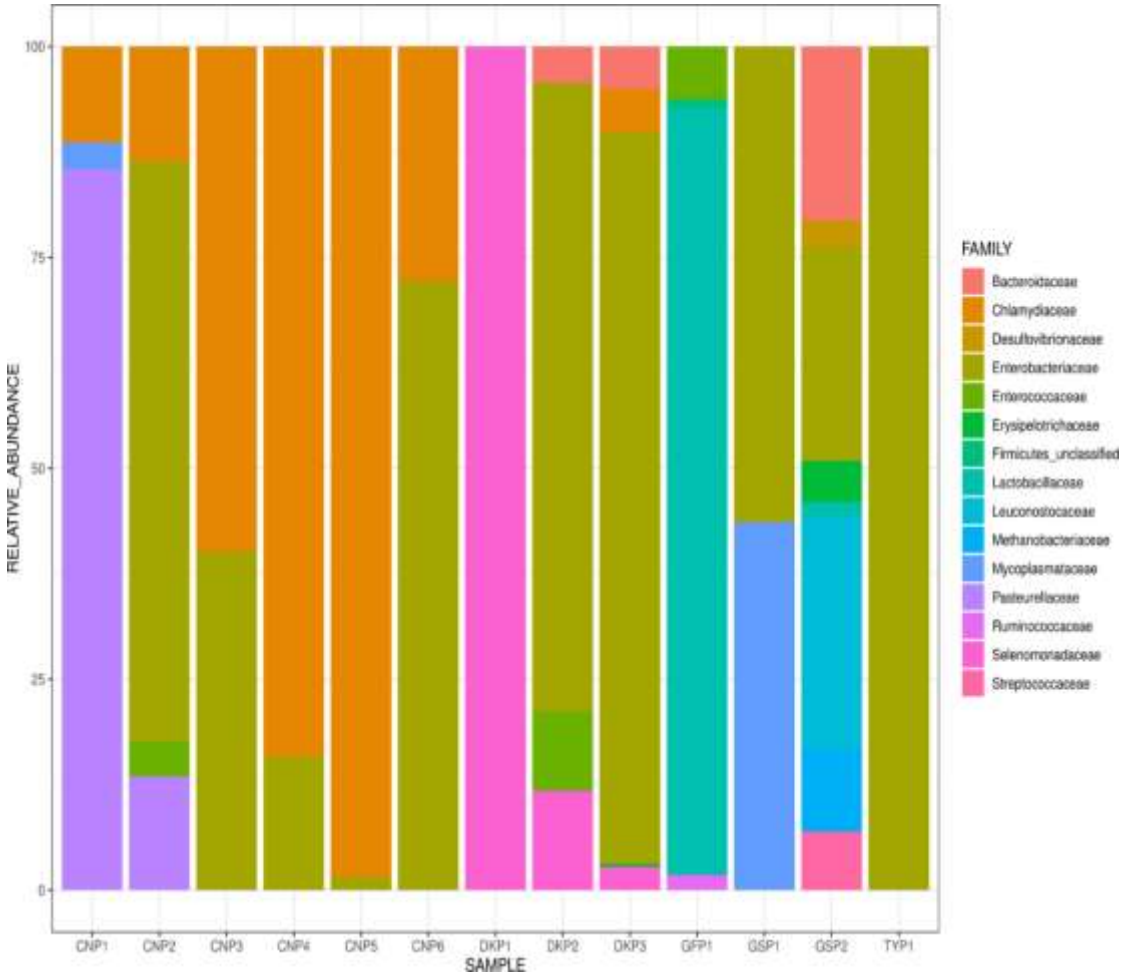


**Figure 4.13: Bacterial Relative Abundance at Family Level (Y-Axis) in all Cloacal Samples (X-Axis).** A stacked column chart with taxonomic relative abundances (y-axis) by sample (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

The relative abundance at the genus level for individual samples are shown below in Figure 4.14. *Desulfovibrio*, *Gallibacterium*, and *Mycoplasma* were the most frequently detected genera across the poultry species. Other genera detected in some poultry included *Escherichia*, *Klebsiella*, *Chlamydia*, *Bacteroides*, and *Avibacterium*. Most of these bacteria are potentially pathogenic.

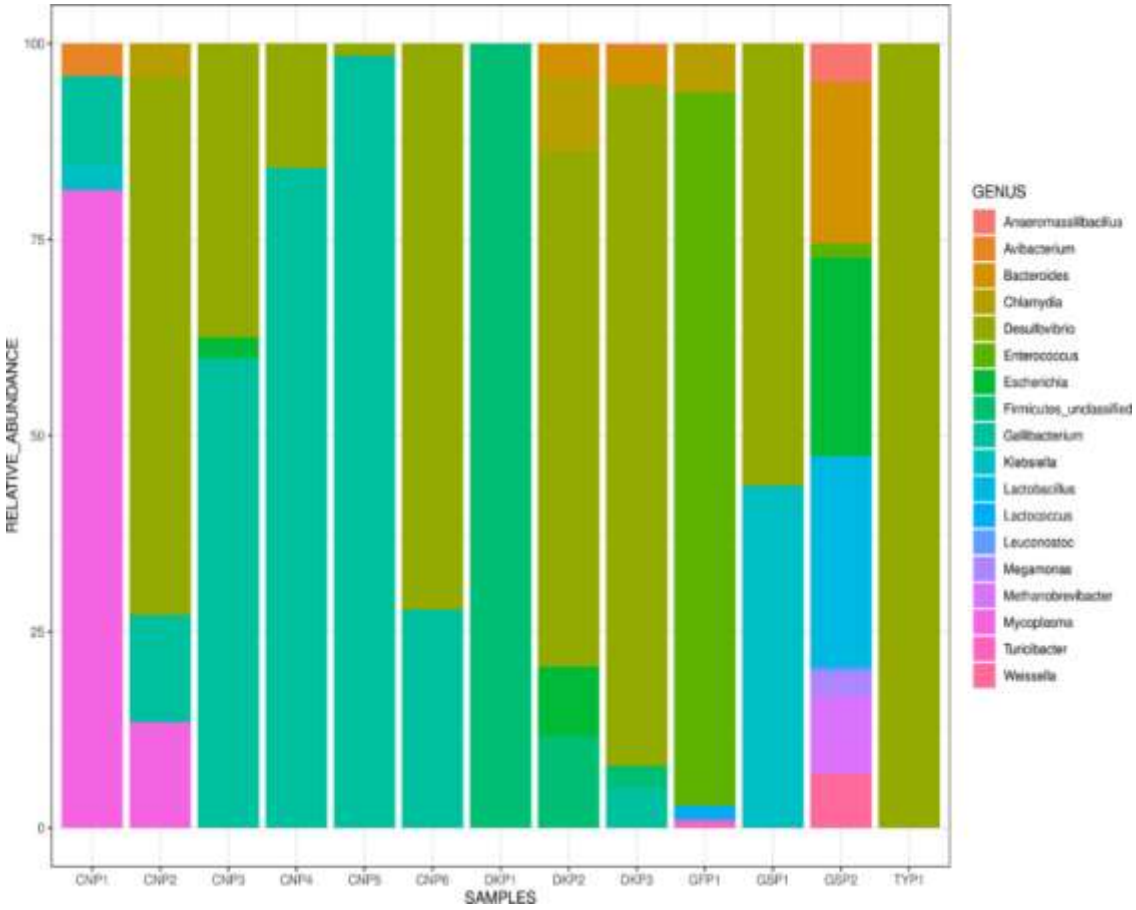


**Figure 4.14: Bacterial Relative Abundance at Genus Level (Y-Axis) in all Cloacal Samples (X-Axis).** A stacked column chart with taxonomic relative abundances (y-axis) by sample (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

Species-level distribution for individual samples are shown below (Figure 4.15). *Escherichia coli* (0.93-100%) and *Chlamydia ibidis* (5.15-98.43%) were the most frequently detected bacterial species across the poultry.

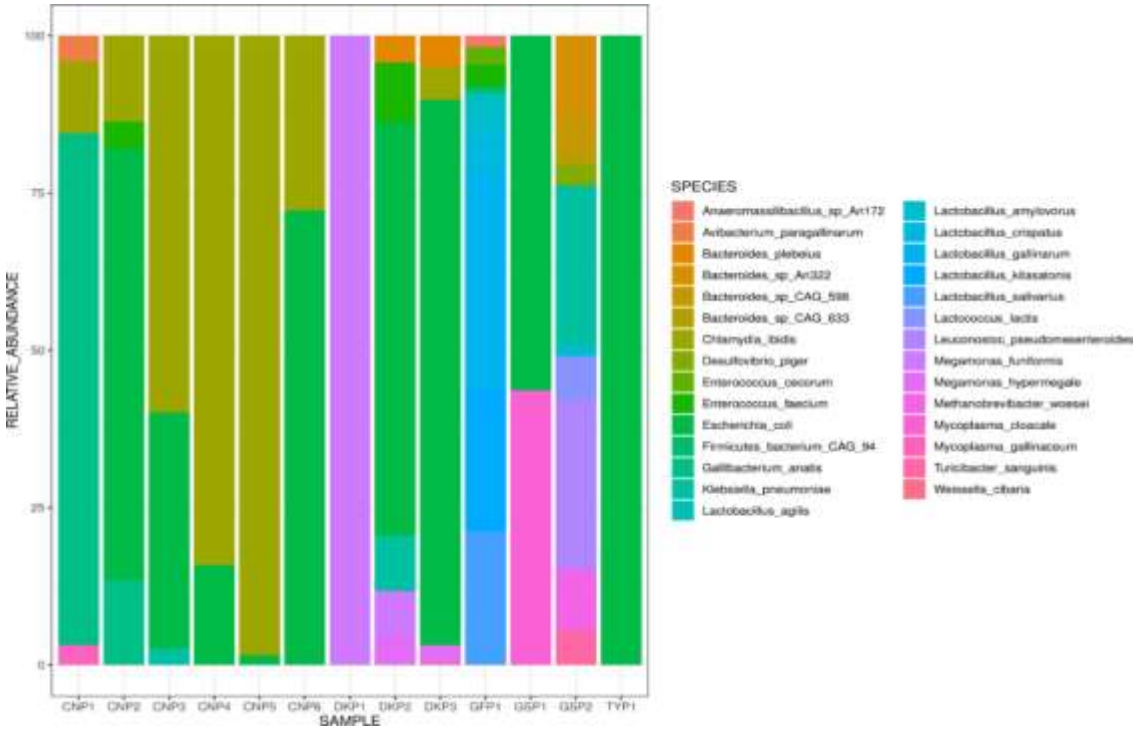


**Figure 4.15: Bacterial Relative Abundance at Species Level (Y-Axis) in all Cloacal Samples (X-Axis).** A stacked column chart with taxonomic relative abundances (y-axis) by sample (x-axis). The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

To assess the relatedness and overall taxonomic similarities between the identified sequences in the cloacal swab samples, a hierarchical clustering analysis of the dominant genera and species of all samples was performed (Figures 4.16 and 4.17). The hierarchical cluster maps for both groups generally had dendrograms with intermingled branches, implying a lack of clear separation between samples from the different poultry. The results therefore, indicate the absence of bacterial pathogen-host specificity for most of the samples studied. The hierarchical cluster maps also showed the dominance of *Desulfovibrio*, *Gallibacterium*, and *Mycoplasma* in the poultry cloacal swab samples. Species abundance was also resolved, revealing that *Escherichia coli* and *Chlamydia ibidis* were the most dominant bacterial species across the poultry.

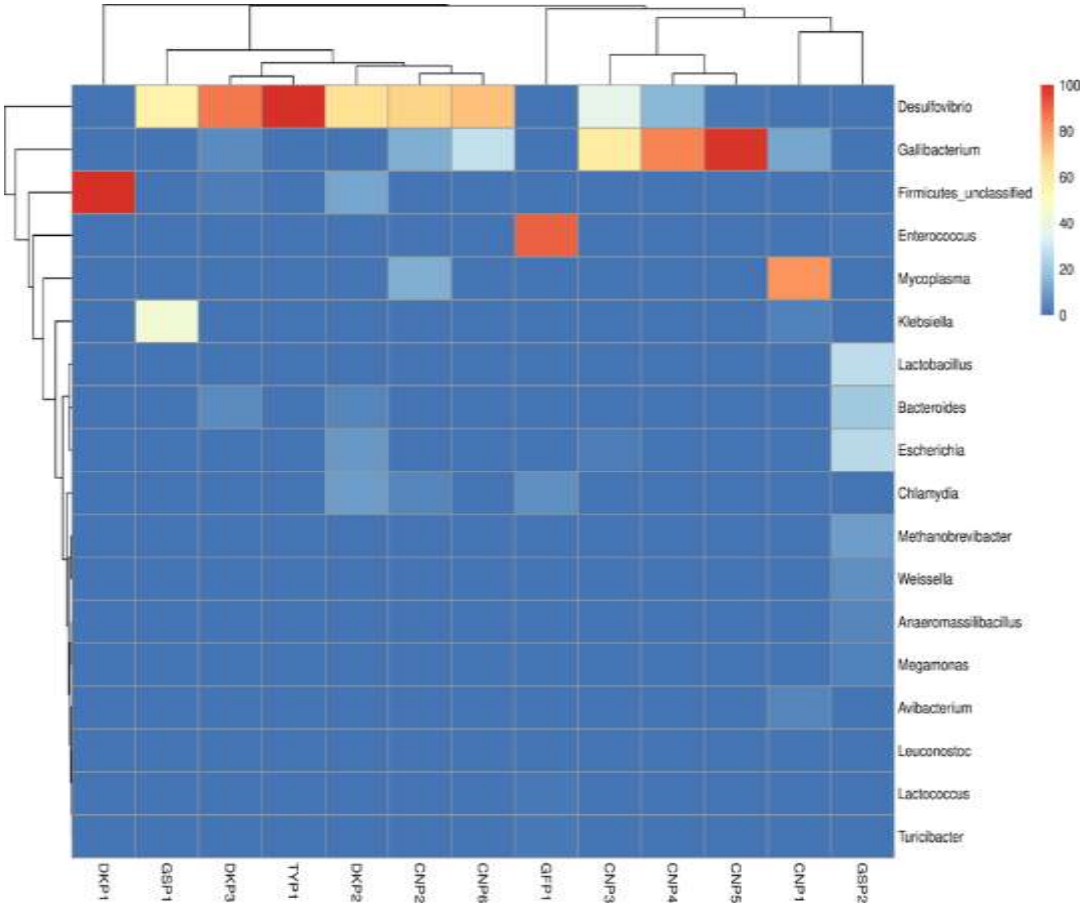


**Figure 4.16: Taxonomic Abundance Heat Map of the Dominant Genera (Y-Axis) in all Cloacal Swab Samples (X-Axis) Based on Log-Transformed Relative Abundance Values.** Colour scale from red (high abundance) to blue (low abundance) represent log-transformed relative abundance. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

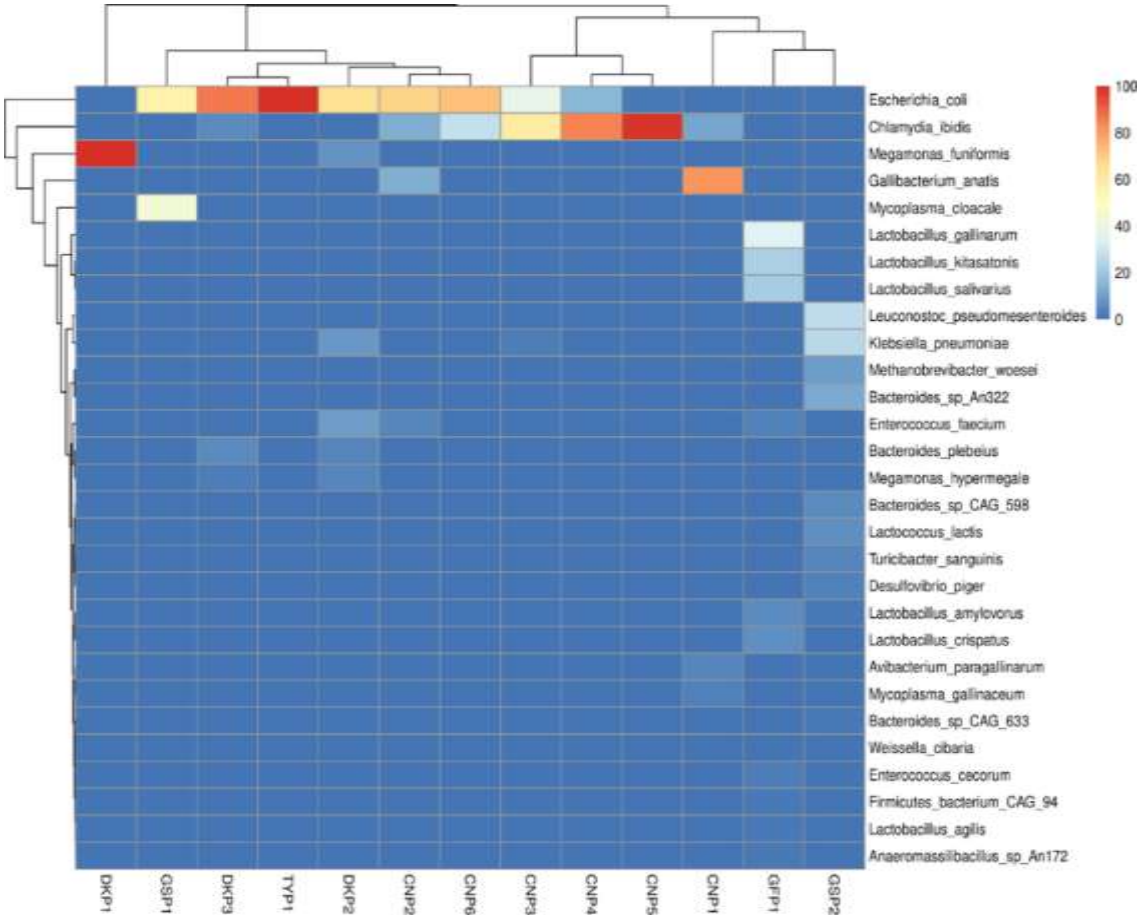


**Figure 4.17: Taxonomic Abundance Heat Map of the Detected Species (Y-Axis) in all Cloacal Swab Samples (X-Axis) Based on Log-Transformed Relative Abundance Values.** Colour scale from red (high abundance) to blue (low abundance) represent log-transformed relative abundance. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

The number of OTUs and the Shannon entropy grouping of the different species and alpha diversity measures by species is shown in the boxplot below (Figures 4.18 and 4.19). The results similarly showed that there was no marked difference in species richness of the detected bacterial pathogens between the different poultry.

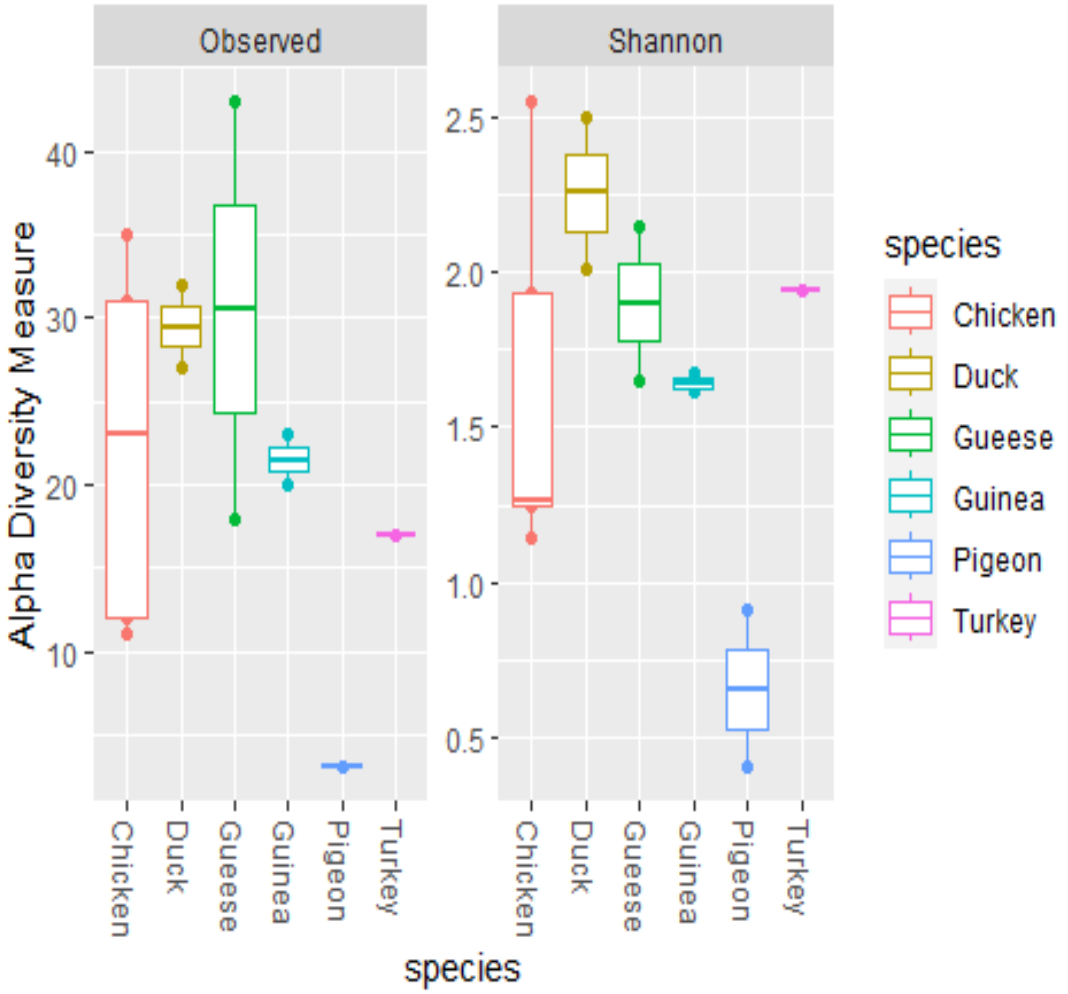


**Figure 4.18: The Number of OTUS and the Shannon Entropy Grouping of the Different Species.**

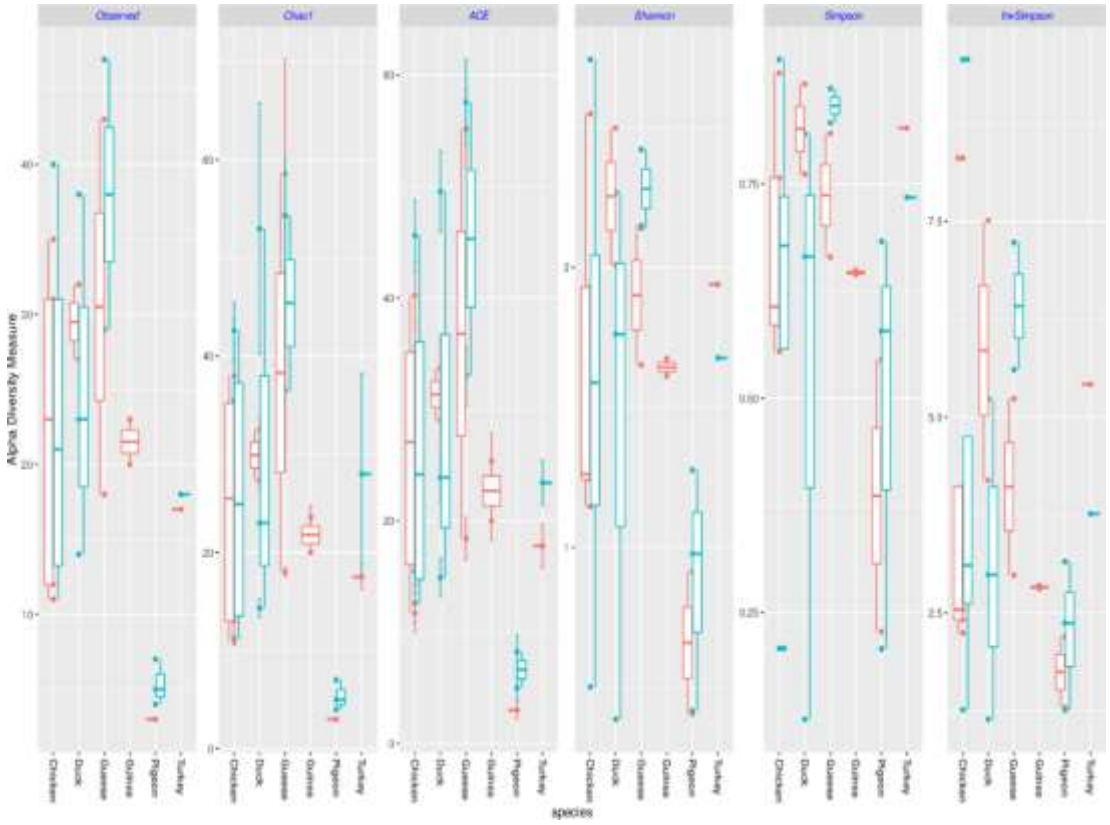


**Figure 4.19: Alpha Diversity Measures by Poultry.**

Diversity indices were also tested using the Wilcoxon rank-sum (Mann-Whitney) non-parametric test to determine if they differed significantly between poultry (Tables 4.6, 4.7, and 4.8). The results show the pairwise comparisons of species richness (Observed, Shannon, and Chao1) between the different poultry species (chicken, duck, goose, guinea fowl, pigeon, and turkey). Based on this diversity indices, no significant difference was observed between any poultry species ($p > 0.05$), hence, the poultry species do not differ significantly in microbiome species richness.

**Table 4.6: Pairwise Comparison of Species Richness between Different Poultry Based on Observed Number of OTUS Using the Holm P Value Adjustment Method**

| | Chicken | Duck | Goose | Guinea fowl | Pigeon |
|---|---|---|---|---|---|
| Duck | 1.00 | - | - | - | - |
| Goose | 1.00 | 1.00 | - | - | - |
| Guinea fowl | 1.00 | 1.00 | 1.00 | - | - |
| Pigeon | 1.00 | 1.00 | 1.00 | 1.00 | - |
| Turkey | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

**Table 4.7: Pairwise Comparison of Species Richness between Different Poultry Based on Shannon Diversity Index Using the Holm P Value Adjustment Method**

| | Chicken | Duck | Goose | Guinea fowl | Pigeon |
|---|---|---|---|---|---|
| Duck | 1.00 | - | - | - | - |
| Goose | 1.00 | 1.00 | - | - | - |
| Guinea fowl | 1.00 | 1.00 | 1.00 | - | - |
| Pigeon | 1.00 | 1.00 | 1.00 | 1.00 | - |
| Turkey | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

**Table 4.8: Pairwise Comparison of Species Richness between Different Poultry Based on Chao1 Diversity Index Using the Holm P Value Adjustment Method**

| | Chicken | Duck | Goose | Guinea fowl | Pigeon |
|---|---|---|---|---|---|
| Duck | 1.00 | - | - | - | - |
| Goose | 1.00 | 1.00 | - | - | - |
| Guinea fowl | 1.00 | 1.00 | 1.00 | - | - |
| Pigeon | 1.00 | 1.00 | 1.00 | 1.00 | - |
| Turkey | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |

The PCoA plot compares the poultry microbiomes by keeping parameter standard error ellipses at 95% confidence level (Figure 4.20). The plot also revealed that there was no clear demarcation between the microbiomes of the different poultry species. Comparison of poultry microbiome using the PCoA plot based on the ordination of the distance matrix was also generated using Bray-Curtis distance (Figure 4.21). Again, a clear demarcation between microbial assemblages from the different poultry was not apparent along the principal coordinate axis 1 (PCO1) of the PCoA plot.

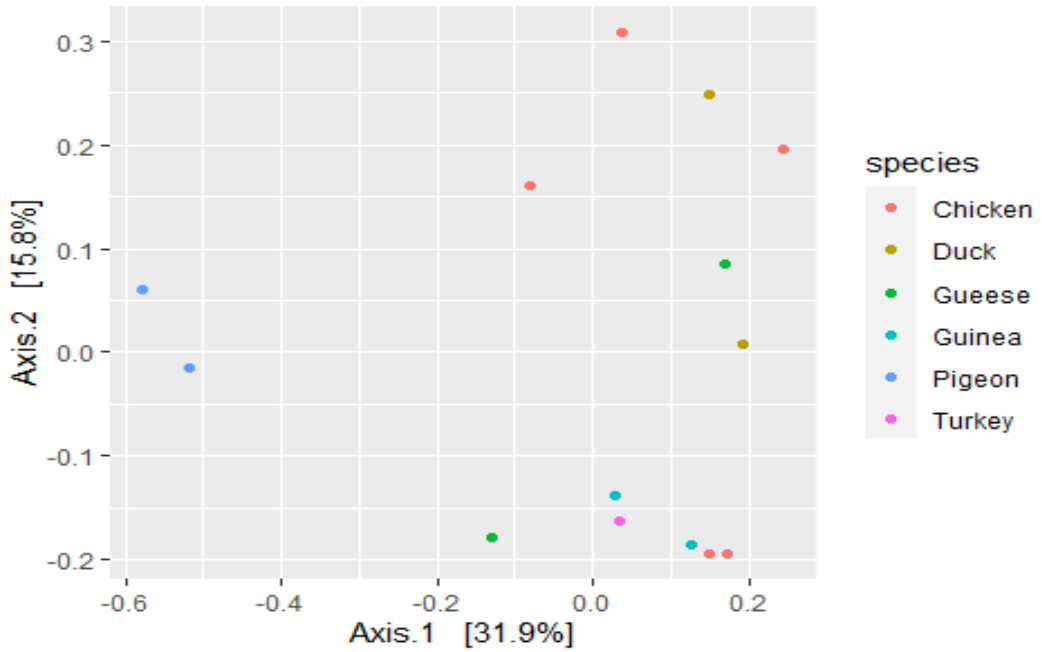


**Figure 4.20: Comparison of Poultry Microbiome Using the Pcoa Plot Based on 97% Similarity Unweighted Unifrac Distance Matrices.**

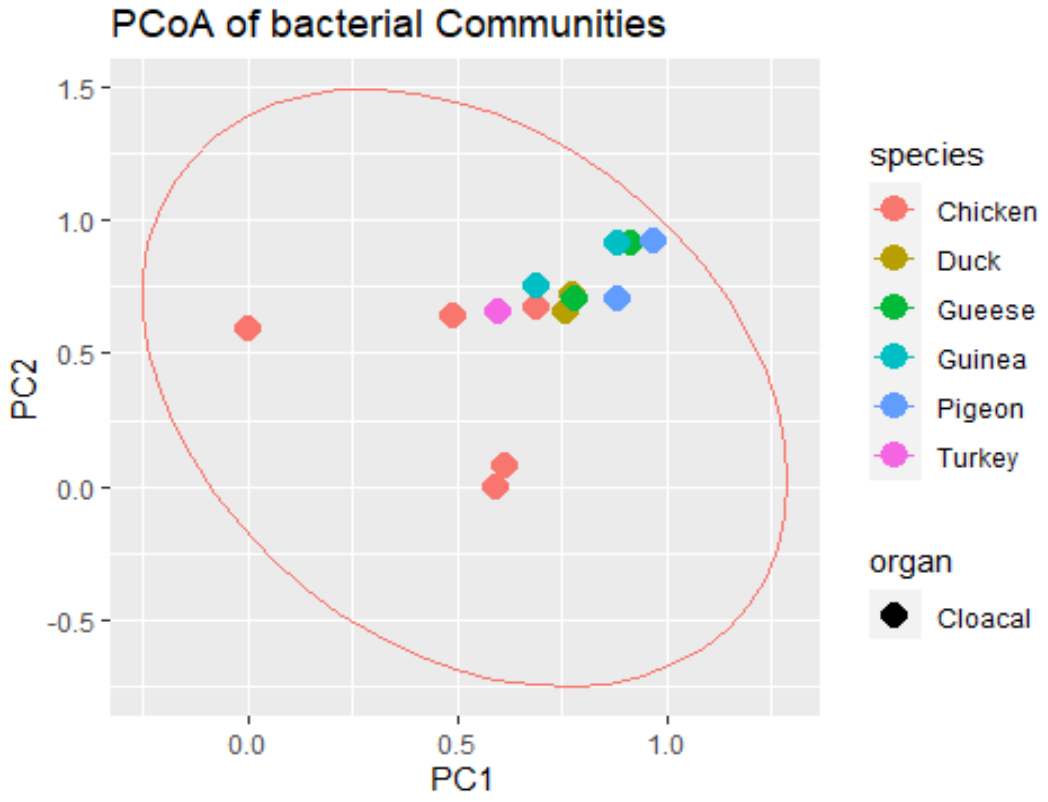


**Figure 4.21: Comparison of Poultry Microbiome Using the Pcoa Plot Based on the Ordination of the Distance Matrix Generated Using Bray-Curtis Distance.**

## 4.4 Evaluation of Gut Microbial Community Profiles

### 4.4.1 General Overview of the Microbiome Sequence Data

A total of 16,795,056 paired-end reads (from faecal samples) and 15,354,006 paired-end reads (from caecal samples), with a median length of 200 base pairs (bp), were obtained from all samples (Tables 4.9 and 4.10). The total number of clean reads generated from faecal and caecal samples were 16,432,416 and 10,879,784, respectively. These were subsequently assembled into a total of 66,090 and 60,098 contigs, respectively. Using a 95% similarity cut-off, the assembled contigs yielded 2,092 and 2,345 operational taxonomic units (OTUs) for faecal and caecal samples, respectively. Three samples (S002, S023, and S027) were not informative as they did not generate any OTUs that could be used for taxonomic assignment.

**Table 4.9: OTUs (0.05% Coverage) and Species Richness from Faecal Samples from Different Poultry**

| Sample | Number of clean reads | Number of contigs | Number of Observed OTUs | Chao1 | ACE | Shannon | Inverse Simpson |
|---|---|---|---|---|---|---|---|
| S001 | 2,616,330 | 5,043 | 143 | 195.56 | 196.91 | 3.443 | 16.26 |
| S003 | 2,092,898 | 6,321 | 138 | 177.00 | 180.45 | 3.232 | 12.94 |
| S005 | 434,508 | 781 | 165 | 220.00 | 233.47 | 3.368 | 14.31 |
| S007 | 166,114 | 182 | 175 | 228.00 | 237.30 | 3.709 | 22.66 |
| S009 | 1,652,376 | 7,304 | 132 | 215.15 | 188.95 | 2.785 | 6.912 |
| S011 | 993,492 | 3,724 | 123 | 168.12 | 177.96 | 1.867 | 2.874 |
| S013 | 1,604,804 | 3,898 | 173 | 252.57 | 246.63 | 3.529 | 19.14 |
| S015 | 748,964 | 3,698 | 157 | 202.04 | 207.36 | 3.609 | 20.11 |
| S017 | 1,347,408 | 12,081 | 144 | 198.67 | 191.68 | 3.599 | 18.86 |
| S019 | 845,996 | 3,529 | 159 | 244.55 | 239.81 | 3.380 | 14.57 |
| S021 | 590,996 | 1,503 | 148 | 229.05 | 225.25 | 3.284 | 12.06 |
| S025 | 1,899,686 | 7,739 | 141 | 171.57 | 175.65 | 2.805 | 6.099 |
| S029 | 817,870 | 7,343 | 145 | 202.50 | 207.51 | 3.711 | 24.83 |
| S031 | 983,614 | 6,099 | 149 | 198.14 | 219.13 | 3.681 | 21.51 |
| Total | 16,795,056 | 69,245 | 2,092 | | | | |

*Odd numbers in the sample IDs indicate faecal samples. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).*

**Table 4.10: OTUs (0.05% Coverage) and Species Richness from Caecal Samples from Different Poultry.**

| Sample | Number of raw reads | Number of contigs | Number of Observed OTUs | Chao1 | ACE | Shannon | Inverse Simpson |
|---|---|---|---|---|---|---|---|
| S004 | 1,515,614 | 1,439 | 158 | 245.00 | 237.52 | 3.085 | 9.202 |
| S006 | 101,244 | 89 | 145 | 179.17 | 185.12 | 3.456 | 17.76 |
| S008 | 368,138 | 862 | 165 | 210.72 | 228.87 | 3.663 | 23.65 |
| S010 | 162,600 | 58 | 169 | 242.20 | 252.33 | 3.438 | 16.49 |
| S012 | 3,470,728 | 20,606 | 176 | 244.44 | 239.06 | 3.500 | 18.24 |
| S014 | 650,780 | 3,571 | 169 | 228.40 | 230.26 | 3.429 | 16.10 |
| S016 | 1,026,998 | 8,639 | 146 | 214.06 | 212.35 | 3.592 | 19.81 |
| S018 | 305,850 | 743 | 148 | 178.10 | 194.32 | 3.206 | 12.07 |
| S020 | 1,172,660 | 6,353 | 155 | 185.88 | 187.80 | 3.483 | 15.67 |
| S022 | 1,070,720 | 5,617 | 133 | 149.61 | 164.46 | 3.381 | 16.75 |
| S024 | 845,972 | 2,331 | 135 | 192.40 | 180.11 | 3.381 | 16.05 |
| S026 | 963,508 | 2,640 | 166 | 168.63 | 173.50 | 3.444 | 10.45 |
| S028 | 1,673,198 | 4,910 | 189 | 262.20 | 260.18 | 3.586 | 14.54 |
| S030 | 1,291,674 | 6,643 | 150 | 189.05 | 195.01 | 3.532 | 17.27 |
| S032 | 734,322 | 6,143 | 141 | 192.48 | 197.55 | 3.340 | 14.80 |
| Total | 15,354,006 | 70,644 | 2,345 | | | | |

*Even numbers in the sample IDs indicate caecal samples. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).*

Rarefaction curves generated from the OTUs show that all the samples approached a plateau, suggesting that the sample volumes were efficient in estimating both faecal and caecal taxa (Figure 4.22).

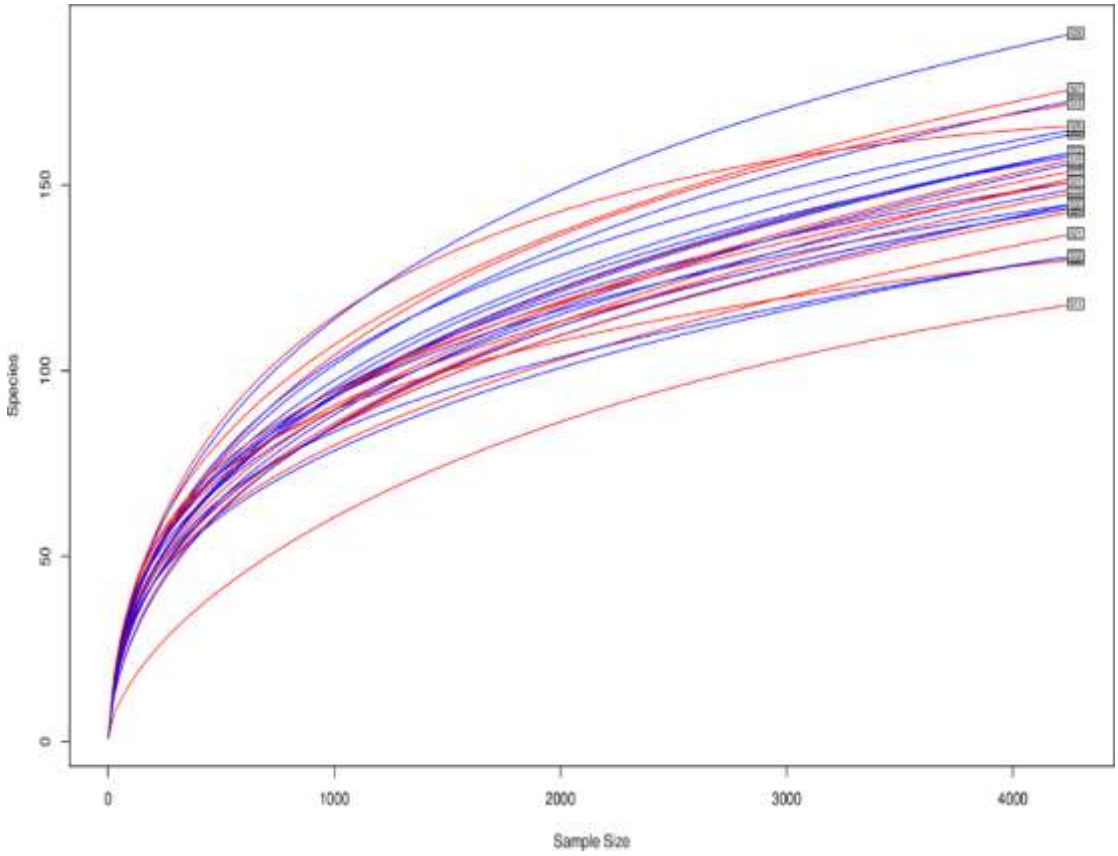


**Figure 4.22: Rarefaction Curves of Samples Clustered at 90% Sequence Identity**. The rarefaction curves for each sample were plotted without replacement. Rarefaction is used to simulate even number of reads per sample. In this study, the rarefaction depth chosen is the 90% of the minimum sample depth in the dataset. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

Analysis of species richness using Chao1, ACE, Shannon, and Inverse Simpson indices showed that there was no significant difference in species richness across the poultry (Tables 4.11 and 4.12).

**Table 4.11: OTUs (0.05% Coverage) and Species Richness from Faecal Samples from Different Poultry.**

| Sample | Number of Observed OTUs | Chao1 | ACE | Shannon | Inverse Simpson |
|---|---|---|---|---|---|
| S001 | 143 | 195.56 | 196.91 | 3.443 | 16.26 |
| S003 | 138 | 177.00 | 180.45 | 3.232 | 12.94 |
| S005 | 165 | 220.00 | 233.47 | 3.368 | 14.31 |
| S007 | 175 | 228.00 | 237.30 | 3.709 | 22.66 |
| S009 | 132 | 215.15 | 188.95 | 2.785 | 6.912 |
| S011 | 123 | 168.12 | 177.96 | 1.867 | 2.874 |
| S013 | 173 | 252.57 | 246.63 | 3.529 | 19.14 |
| S015 | 157 | 202.04 | 207.36 | 3.609 | 20.11 |
| S917 | 144 | 198.67 | 191.68 | 3.599 | 18.86 |
| S019 | 159 | 244.55 | 239.81 | 3.380 | 14.57 |
| S021 | 148 | 229.05 | 225.25 | 3.284 | 12.06 |
| S025 | 141 | 171.57 | 175.65 | 2.805 | 6.099 |
| S029 | 145 | 202.50 | 207.51 | 3.711 | 24.83 |
| S031 | 149 | 198.14 | 219.13 | 3.681 | 21.51 |
| Total | 2,092 | | | | |

*Odd numbers in the sample IDs indicate faecal samples. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).*

**Table 4.12: OTUs (0.05% Coverage) and Species Richness from Caecal Samples from Different Poultry.**

| Sample | Number of Observed OTUs | Chao1 | ACE | Shannon | Inverse Simpson |
|---|---|---|---|---|---|
| S004 | 158 | 245.00 | 237.52 | 3.085 | 9.202 |
| S006 | 145 | 179.17 | 185.12 | 3.456 | 17.76 |
| S008 | 165 | 210.72 | 228.87 | 3.663 | 23.65 |
| S010 | 169 | 242.20 | 252.33 | 3.438 | 16.49 |
| S012 | 176 | 244.44 | 239.06 | 3.500 | 18.24 |
| S014 | 169 | 228.40 | 230.26 | 3.429 | 16.10 |
| S016 | 146 | 214.06 | 212.35 | 3.592 | 19.81 |
| S018 | 148 | 178.10 | 194.32 | 3.206 | 12.07 |
| S020 | 155 | 185.88 | 187.80 | 3.483 | 15.67 |
| S022 | 133 | 149.61 | 164.46 | 3.381 | 16.75 |
| S024 | 135 | 192.40 | 180.11 | 3.381 | 16.05 |
| S026 | 166 | 168.63 | 173.50 | 3.444 | 10.45 |
| S028 | 189 | 262.20 | 260.18 | 3.586 | 14.54 |
| S030 | 150 | 189.05 | 195.01 | 3.532 | 17.27 |
| S032 | 141 | 192.48 | 197.55 | 3.340 | 14.80 |
| Total | 2,345 | | | | |

*Even numbers in the sample IDs indicate caecal samples. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).*

### 4.4.1 Faecal and Caecal Microbiome Composition

Phylum-level distributions for individual faecal and caecal samples are shown (Figures 4.23 and 4.24, respectively). In total, 13 phyla were identified in the faecal samples. *Bacteroidetes* (1.60-93.48%), *Firmicutes* (3.22-48.40%), *Proteobacteria* (2.57-34.64%), and *Euryarchaeota* (0.32-22.72%) were the most frequently detected phyla in faecal samples across the poultry. There were 15 phyla were identified in the faecal and caecal samples. *Bacteroidetes* (3.31-90.85%), *Firmicutes* (12.90-67.28%) and *Proteobacteria* (1.18-85.93%) were the most frequently detected phyla

in caecal samples across the poultry. Other phyla detected, albeit in lower proportions were *Euryarchaeota*, *Synergistetes*, *Spirochaetes*, *Chlamydia*, *Actinobacteria*, and *Lentisphaerae*, among others. Chicken faecal and caecal samples had the highest number of phyla compared to the other poultry.

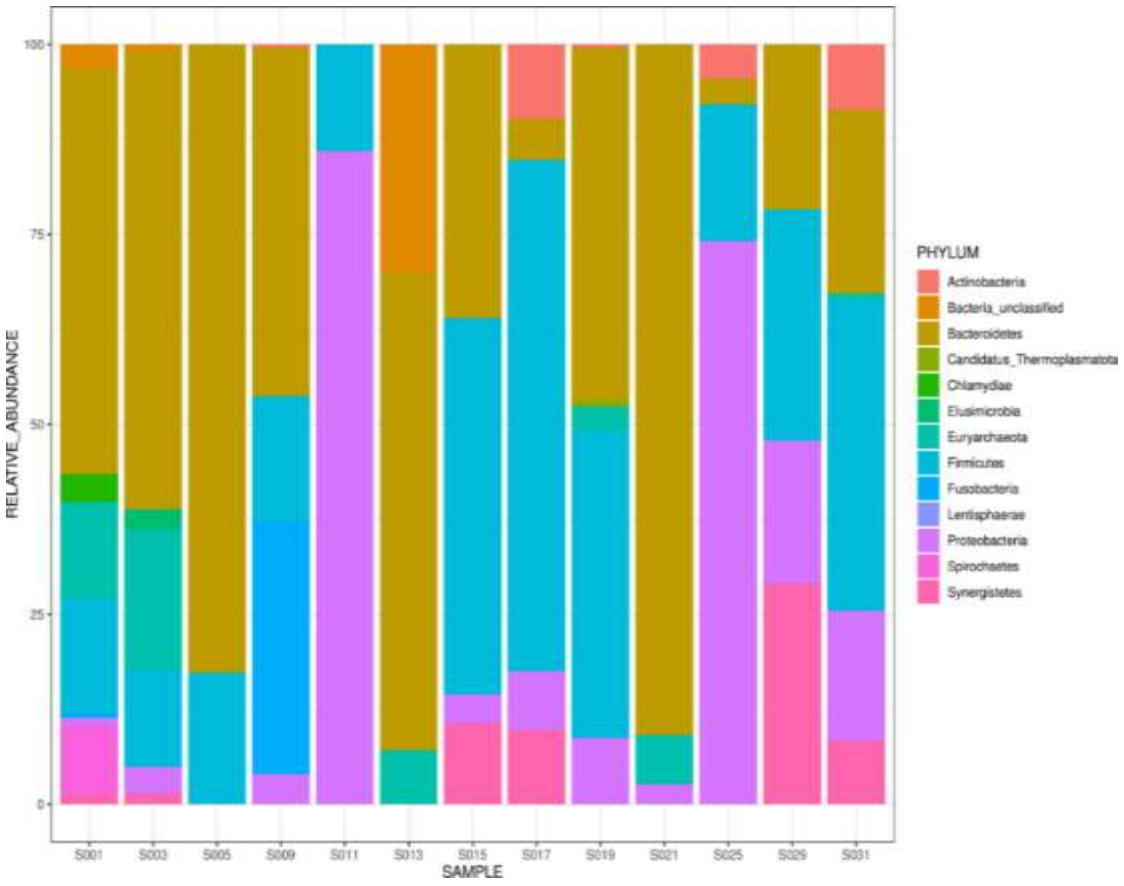


**Figure 4.23: Faecal Relative Abundance at Phylum Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

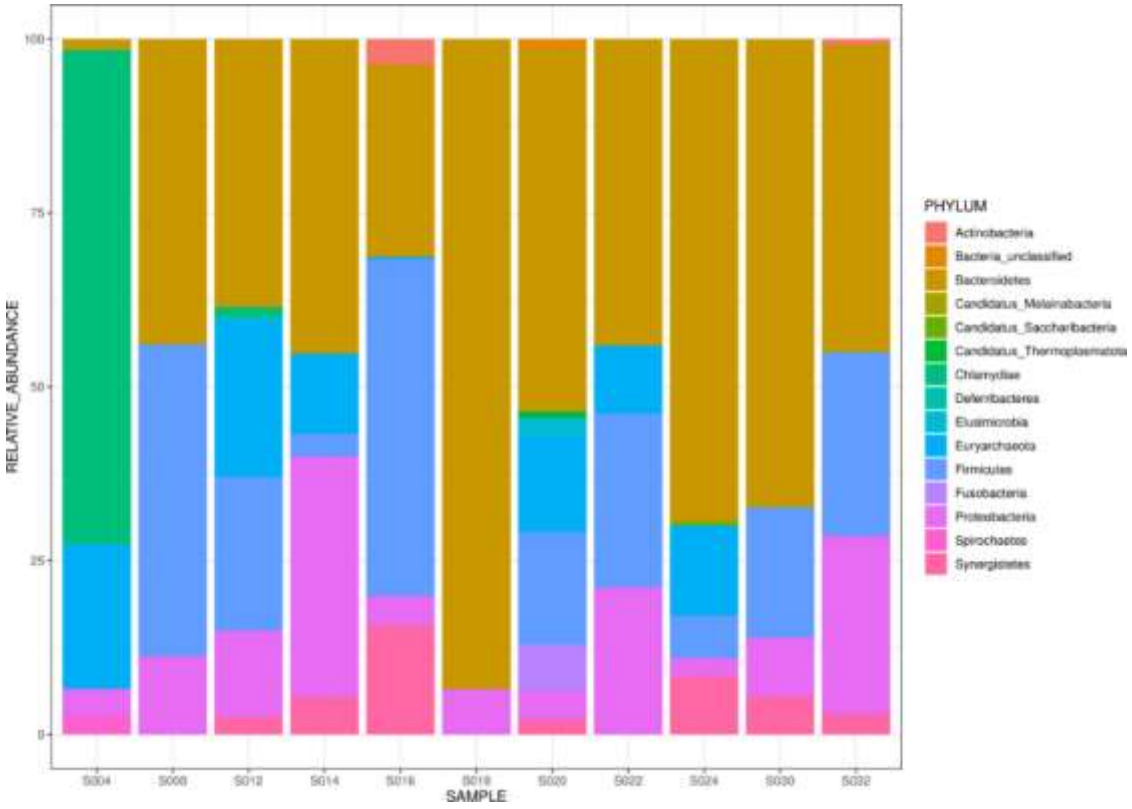


**Figure 4.24: Caecal Relative Abundance at Phylum Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

Class-level distributions for individual faecal and caecal samples are shown (Figure 4.25 and 4.26, respectively). There were 21 classes of microbes found in faecal samples across the different poultry, with *Bacteoidia* (3.31-82.96%), *Clostridia* (5.13-59%), and *Methanobacteria* (0.1-18.4%) being the most frequently detected in chickens, ducks and guineafowls. Similarly, there were 21 classes of microbes found in caecal samples across the different poultry, with *Bacteoidia* (0.79-91.61%), *Clostridia* (2.5-26.76%), and *Methanobacteria* (0.3-22.95%) being the most frequently detected in chickens, ducks and guineafowls. Others classes that were detected included *Gammaproteobacteria*, *Epsilonproteobacteria*, *Bacilli*, *Deltaproteobacteria*, *Betaproteobacteria*, *Chlamydia*, *Spirochaetia*, and *Synergistia*, among others.

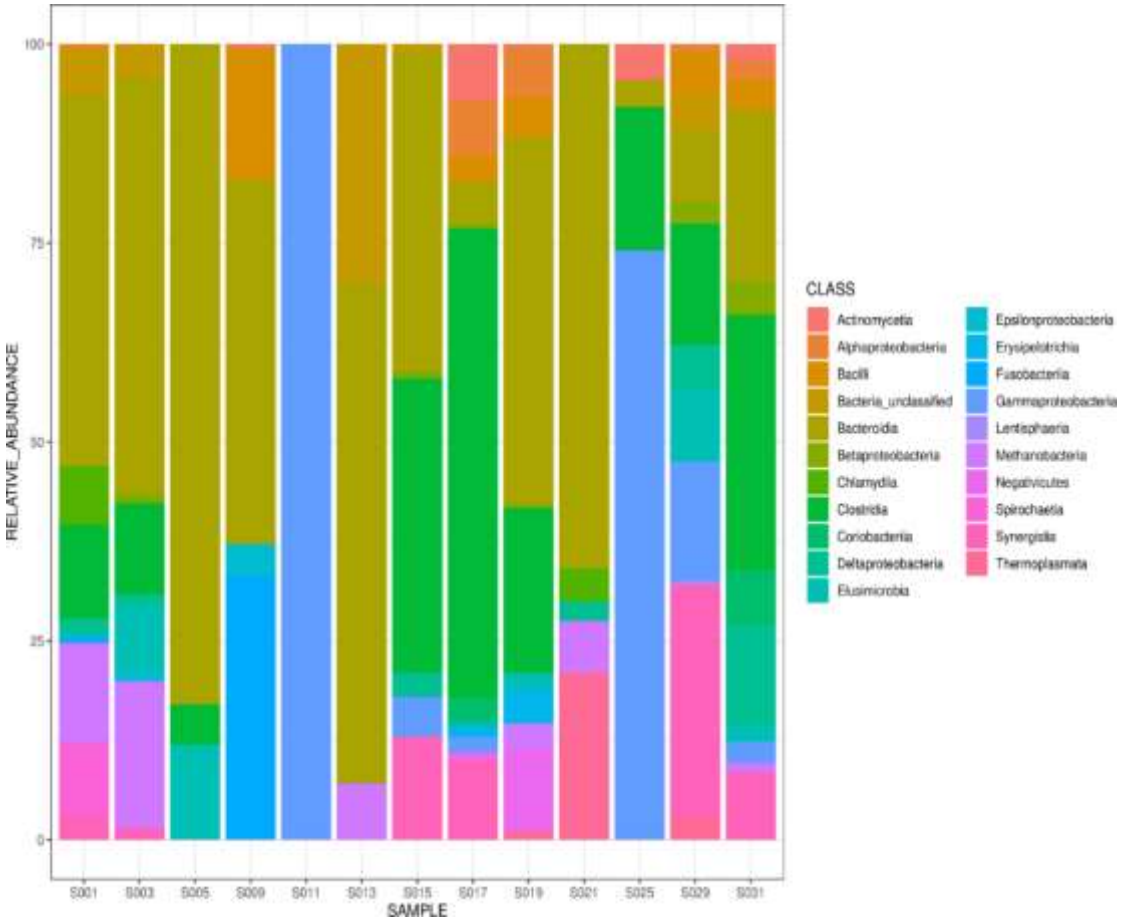


**Figure 4.25: Faecal Relative Abundance at Class Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

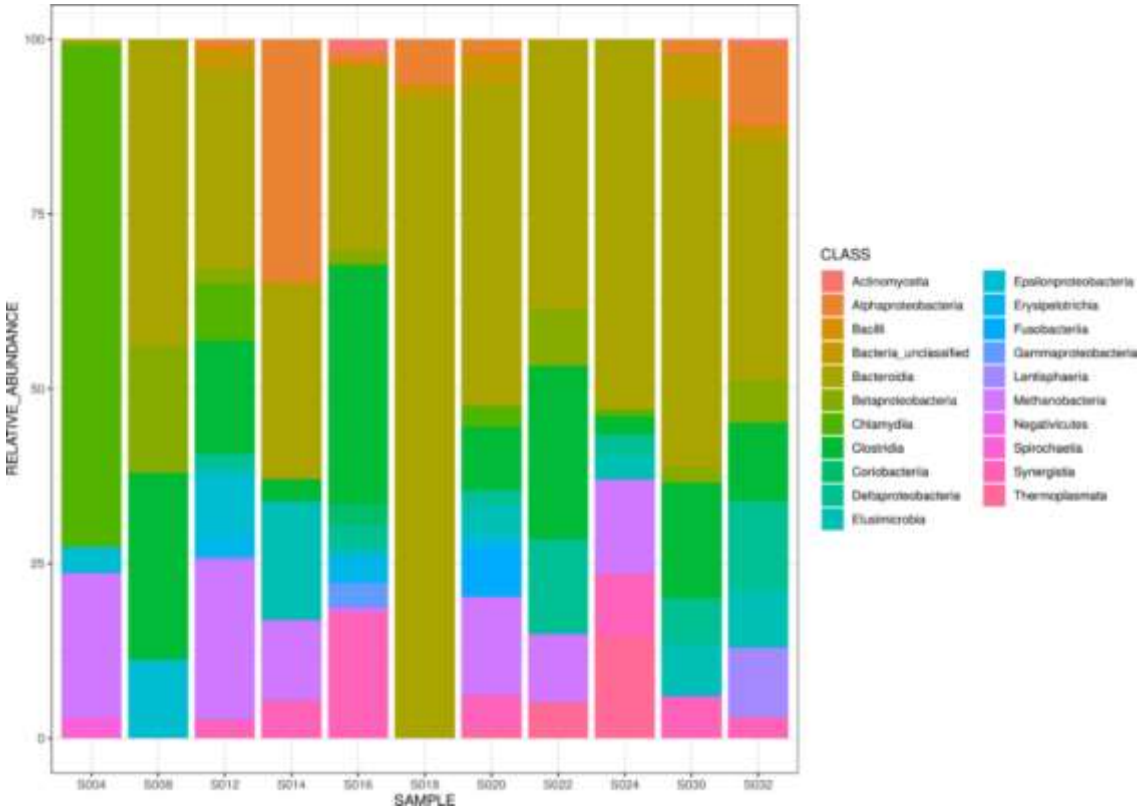


**Figure 4.26: Caecal Relative Abundance at Class Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

Family-level distributions for individual faecal and caecal samples are shown (Figure 4.27 and 4.28, respectively). At the family level, 37 and 33 taxa were identified in faecal and caecal samples respectively across the poultry. The most frequently detected families in faecal samples were *Bacteroidaceae* (3.05-82.2%), *Oscillospiraceae* (2.54-56%), and *Methanobacteriaceae* (0.15-18.4%). Similarly, the most frequently detected families in caecal samples across the poultry were *Bacteroidaceae* (0.79-91.6%), *Oscillospiraceae* (0.35-25.35%), and *Methanobacteriaceae* (0.35-23%). Others included *Lachnospiracaea*, *Clostridiaceae*, *Prevotellaceae*, *Synergistaceae*, and *Chlamydiacaea*, among others.

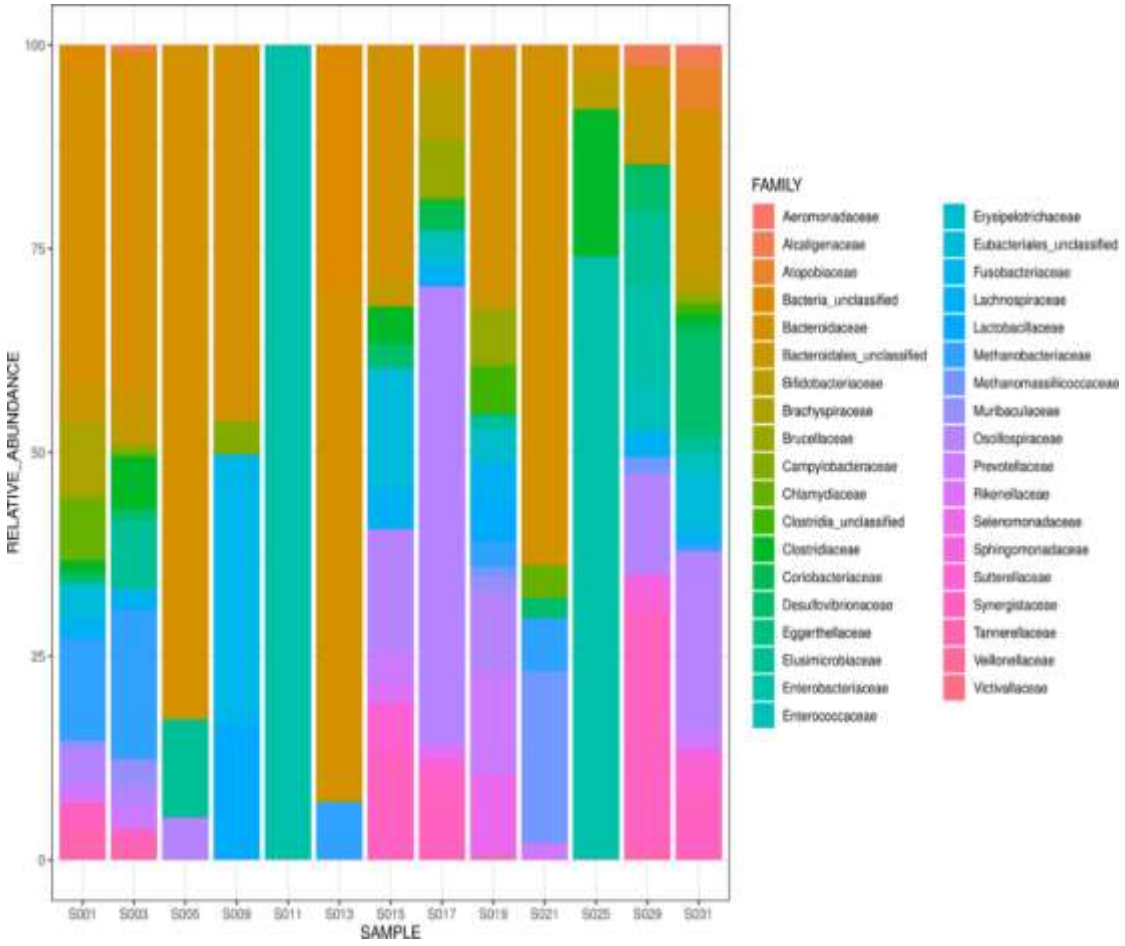


**Figure 4.27: Faecal Relative Abundance at Family Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

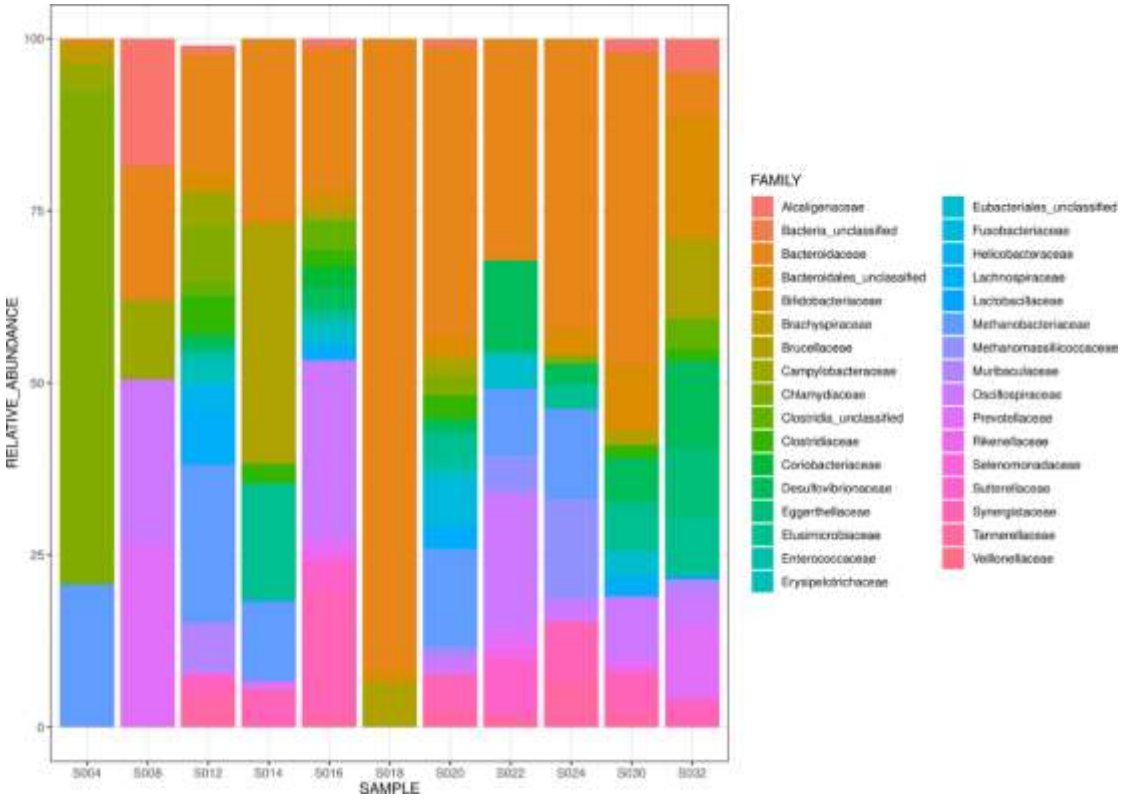


**Figure 4.28: Caecal Relative Abundance at Family Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

Genus-level distributions for individual faecal and caecal samples are shown (Figure 4.29 and 4.30, respectively). A total of 61 and 57 genera were identified in faecal and caecal samples, respectively, across the poultry. *Bacteroides* (0.27-52.43%), *Pseudoflavonifractor* (1.96-27.54%), *Faecalibacterium* (0.08-20.61), *Methanobrevibacter* (0.06-18.35%), *Elusimicrobium* (1.38-15.85), *Prevotela* (1.23-9.13), *Chlamydia* (0.27-7.37%), and *Candidatus Alloclostridium* (0.34-6.15%), were the most frequently detected genera in poultry caecal samples. The dominant genera in the faecal samples were *Bacteroides* (0.30-48.37%), *Phocaeicola* (1.21-35.17%), *Methanobrevibacter* (0.32-22.72%), *Candidatus Adamsella* (0.76-16.85%), *Pseudoflavonifractor* (0.29-15.91), and *Mediterranea* (0.22-8.75%), and among others.

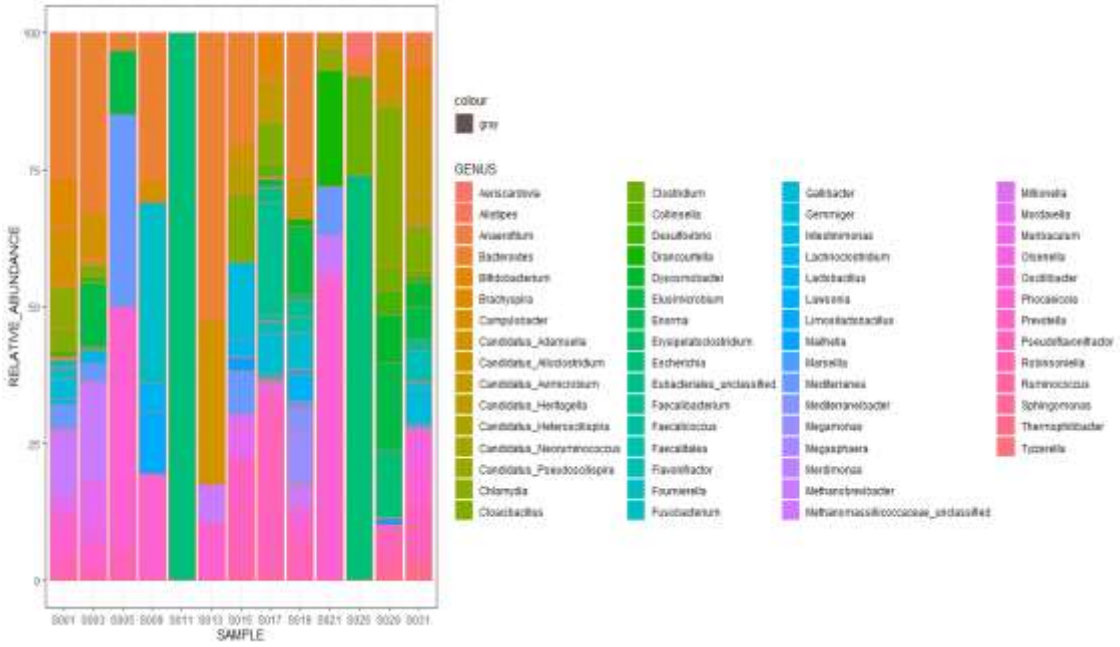


**Figure 4.29: Faecal Relative Abundance at Genus Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

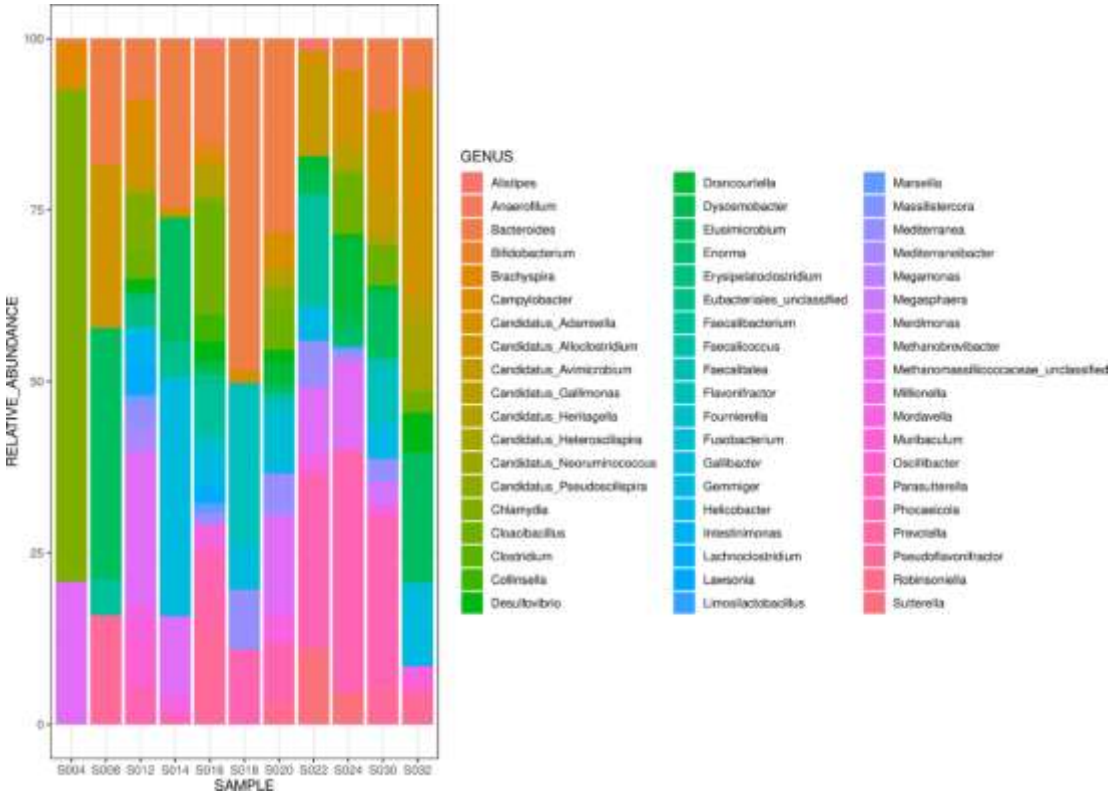


**Figure 4.30: Caecal Relative Abundance at Genus Level (Y-Axis) in all Samples (X-Axis).** The height of each bar chart relates to the taxonomic relative abundances in a sfsdaample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

There were 95 and 89 species found in both faecal and caecal samples, respectively, across the different poultry (data not shown). The most frequently detected microbial species in faecal samples were *Phocaeicola barnesiae* (0.20-45.38%), *Bacteroides* sp. An322 (0.08-33.63%), *Bacteroides caecigallinarum* (0.27-25.12%), and *Methanobrevibacter woesei* (0.06-18.35%). Similarly, the most dominant microbial species in caecal samples were *Chlamydia gallinacea* (0.44-71.82%), *Methanobrevibacter woesei* (0.32-22.72%), *Phocaeicola barnesiae* (1.06-21.18%), *Bacteroides caecicola* (1.91-18.44%), and *Bacteroides caecigallinarum* (0.79-10.43%).

To assess the relatedness and overall taxonomic similarities between the identified sequences in the faecal and caecal samples, a hierarchical clustering analysis of the dominant genera and species of all samples for both groups was performed (Figures

4.31 and 4.32). The hierarchical cluster maps for both groups generally had dendrograms with intermingled branches, implying a lack of clear separation between samples from the different poultry. The results therefore indicate a lack of host specificity for most of the samples studied. However, certain microorganisms were only detected in the faeces and not the caecum, and vice versa. The hierarchical cluster maps also showed the dominance of *Bacteroides*, *Pseudoflavonifractor*, *Faecalibacterium*, *Methanobrevibacter*, *Elusimicrobium*, *Prevotela*, *Chlamydia*, and *Candidatus Alloclostridium* in the poultry faeces. Additionally, the cluster map also showed that *Bacteroides*, *Phocaeicola*, *Methanobrevibacter*, *Candidatus Adamsella*, *Pseudoflavonifractor*, and *Mediterranea* were the most dominant genera in the the poultry caeca. Species abundance was also resolved (data not shown), revealing that *Phocaeicola barnesiae*, *Bacteroides* sp. An322, *Bacteroides caecigallinarum*, and *Methanobrevibacter woesei* were the most frequently detected microbial species in the poultry faeces, while *Chlamydia gallinacea*, *Methanobrevibacter woesei*, *Phocaeicola barnesiae*, *Bacteroides caecicola*, and *Bacteroides caecigallinarum* dominated the poultry caecum.

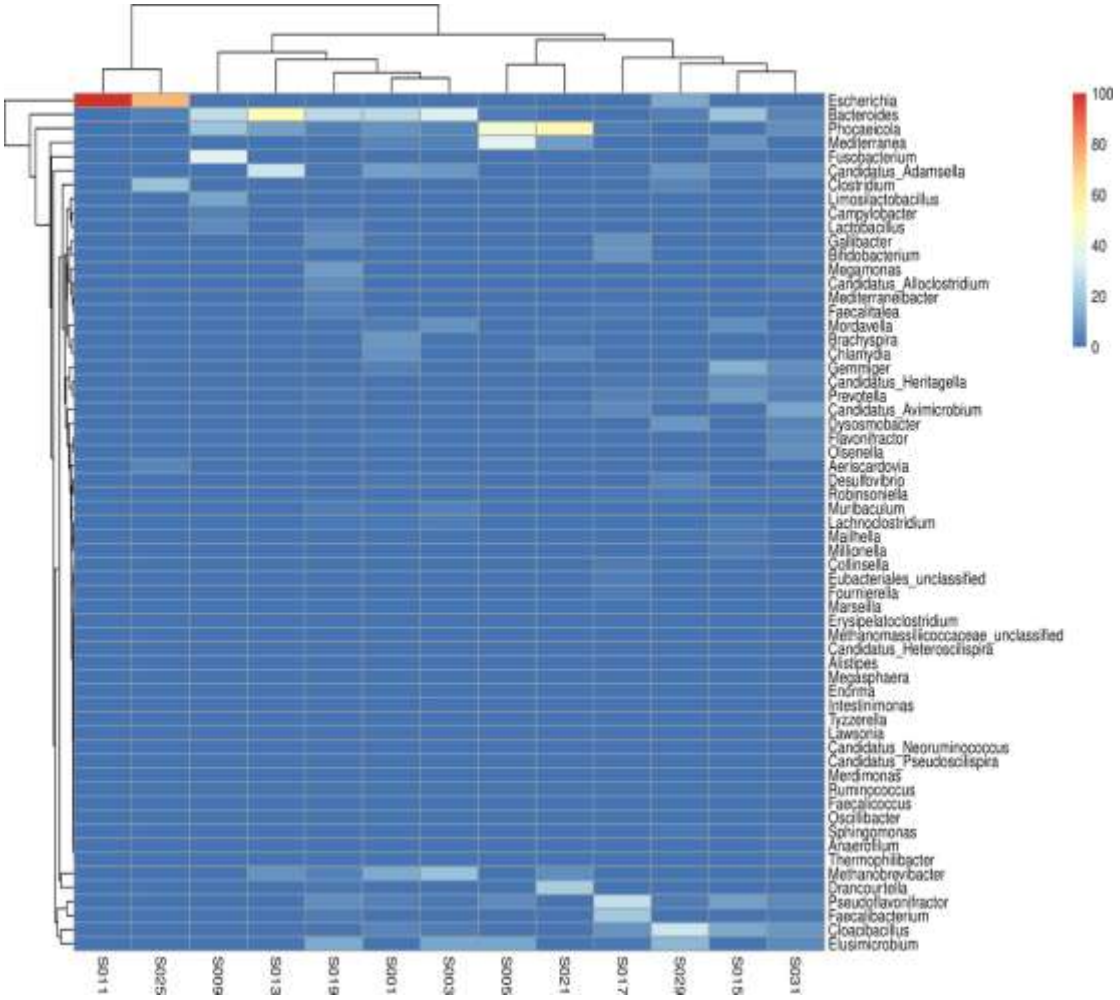


**Figure 4.31: Taxonomic Abundances Heat Map of Faecal Taxonomy Abundance of the Dominant Genera (Y-Axis) in all Samples (X-Axis) Based on Log-Transformed Relative Abundance Values.** The colours scale from red (high abundance) to blue (low abundance) represent log-transformed relative abundance. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

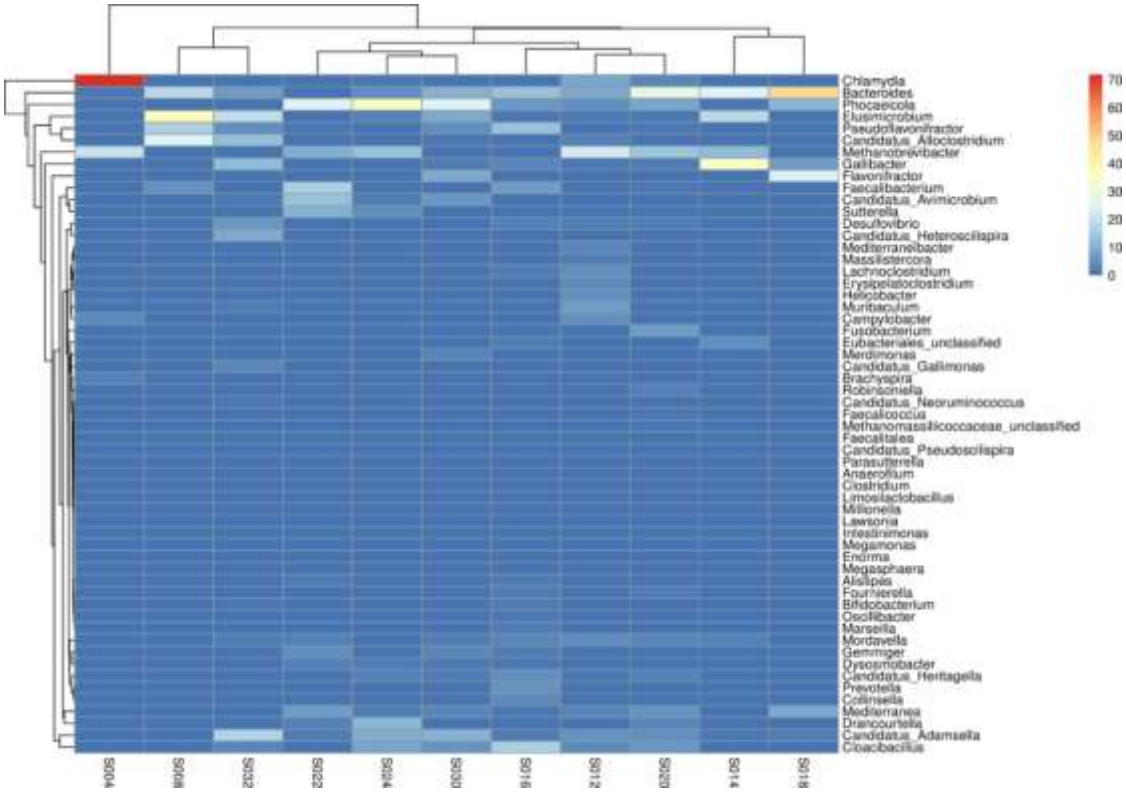


**Figure 4.32: Taxonomic Abundances Heat Map of Caecal Taxonomy Abundance of the Dominant Genera (Y-Axis) in all Samples (X-Axis) Based on Log-Transformed Relative Abundance Values.** The colours scale from red (high abundance) to blue (low abundance) represent log-transformed relative abundance. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

The number of OTUs and the Shannon entropy grouping the different species by sample type, poultry species, and alpha diversity indices are shown in boxplots below (Figures 4.33, 4.34, and 4.35 respectively). The results showed that there was no marked difference in species richness of the detected bacterial pathogens between the different poultry.

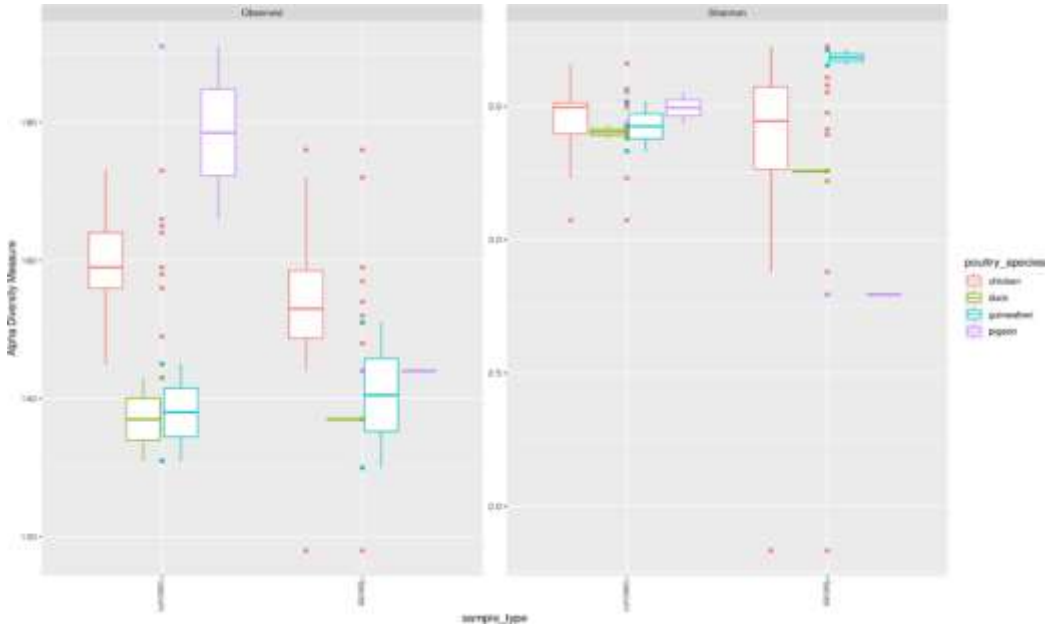


**Figure 4.33: Boxplot of the Number of OTUS and The Shannon Entropy Grouping of the Different Species by Sample Type.**

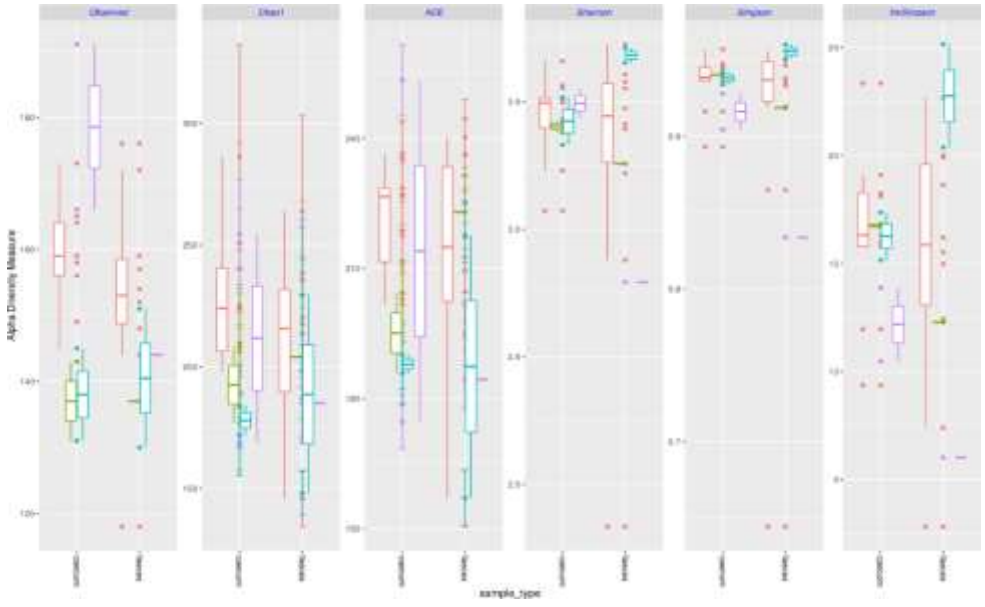


**Figure 4.34: Boxplot of Alpha Diversity Measures by Sample Type.**

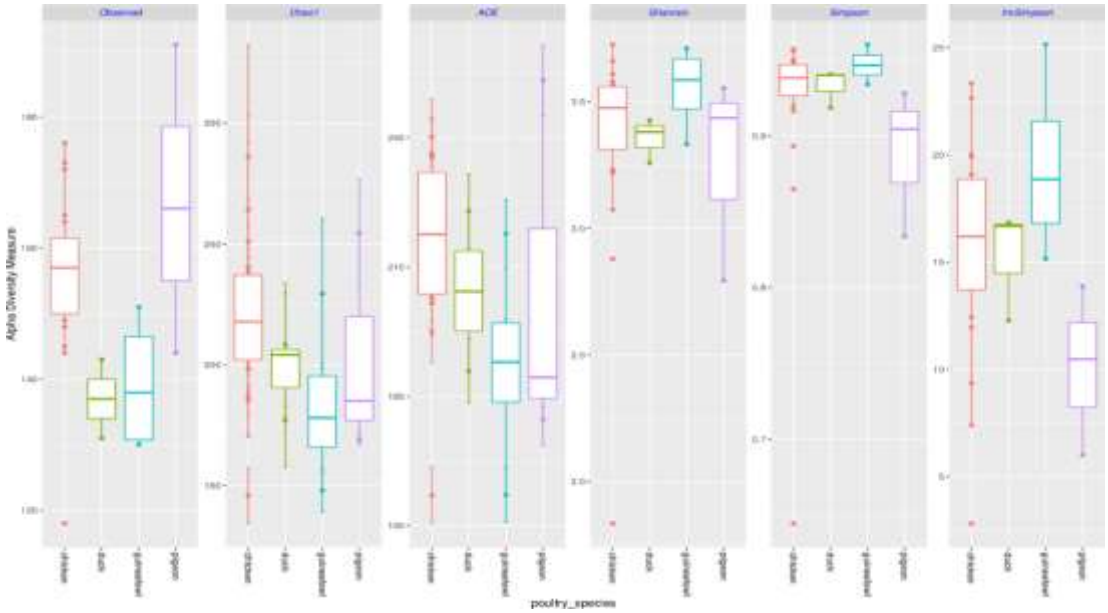


**Figure 4.35: Boxplot of Alpha Diversity Measures by Poultry.**

To test if the metagenome species richness differed significantly between sample types, the non-parametric Wilcoxon rank-sum (Mann-Whitney) test was used (Tables 4.13, 4.14, and 4.15). The pairwise comparisons using the Wilcoxon rank sum test with continuity correction showed that the differences in metagenome species richness between faecal and caecal samples were not significant ($p > 0.05$).

**Table 4.13: Pairwise Comparison of Poultry Metagenomes in Different Sample Types Using Mann-Whitney Test Based on Observed Number of OTUs**

| | **Caecum** |
|---|---|
| **Faeces** | 0.25 |

**Table 4.14: Pairwise Comparison of Poultry Metagenomes in Different Sample Types Using Mann-Whitney Test Based on Shannon Diversity Index**

| | **Caecum** |
|---|---|
| **Faeces** | 0.88 |

**Table 4.15: Pairwise Comparison of Poultry Metagenomes in Different Sample Types Using Mann-Whitney Test Based on Chao1 Diversity Index**

| | **Caecum** |
|---|---|
| **Faeces** | 0.75 |

The metagenome species richness was also tested to determine if it differed significantly between different poultry using the non-parametric Mann-Whitney test (Tables 4.16, 4.17, and 4.18). The results show the pairwise comparisons of species richness (Observed, Shannon, and Chao1) between different poultry (Chicken, Duck, Pigeon, and Guinea fowl). Based on these diversity indices, no significant difference was observed between any poultry ($p > 0.05$). Hence, the different poultry types do not differ significantly in microbiome species richness.

**Table 4.16: Pairwise Comparison of Metagenome Species Richness between Different Poultry Based on Observed Number of Otus. P-Value Adjustment Method: Holm**

| | **Chicken** | **Duck** | **Guinea fowl** |
|---|---|---|---|
| Duck | 0.68 | - | - |
| Guinea fowl | 1.00 | 1.00 | - |
| Pigeon | 1.00 | 1.00 | 1.00 |

**Table 4.17: Pairwise Comparison of Metagenome Species Richness between Different Poultry Based on Shannon Diversity Index. P-Value Adjustment Method: Holm**

| | **Chicken** | **Duck** | **Guinea fowl** |
|---|---|---|---|
| Duck | 1.00 | - | - |
| Guinea fowl | 0.97 | 1.00 | - |
| Pigeon | 1.00 | 1.00 | 1.00 |

**Table 4.18: Pairwise Comparison of Metagenome Species Richness between Different Poultry Based on Chao1 Diversity Index. P-Value Adjustment Method: Holm**

| | Chicken | Duck | Guinea fowl |
|---|---|---|---|
| Duck | 1.00 | - | - |
| Guinea fowl | 1.00 | 1.00 | - |
| Pigeon | 1.00 | 1.00 | 1.00 |

Pairwise comparisons using the Wilcoxon rank sum test with continuity correction and the Wilcoxon rank sum exact test were also used to test whether the species richness (the observed number of OTUs and the Shannon and Chao1 diversity indices) differed significantly between regions for the different poultry species (Tables 4.19, 4.20, and 4.21). Similarly, no significant difference was observed in the metagenomes of the poultry sampled from the different regions ($p > 0.05$).

**Table 4.19: Pairwise Comparison of Metagenome Species Richness between Different Regions Based on Observed Number of Otus. P Value Adjustment Method: Holm**

| | Bungoma | Kilifi | Kwale | Siaya |
|---|---|---|---|---|
| Kilifi | 1.00 | - | - | - |
| Kwale | 1.00 | 1.00 | - | - |
| Siaya | 1.00 | 1.00 | 1.00 | - |
| Turkana | 1.00 | 1.00 | 1.00 | 1.00 |

**Table 4.20: Pairwise Comparison of Metagenome Species Richness between Different Regions Based on Shannon Diversity Index. P Value Adjustment Method: Holm**

| | **Bungoma** | **Kilifi** | **Kwale** | **Siaya** |
|---|---|---|---|---|
| Kilifi | 1.00 | - | - | - |
| Kwale | 1.00 | 1.00 | - | - |
| Siaya | 1.00 | 1.00 | 1.00 | - |
| Turkana | 1.00 | 1.00 | 1.00 | 1.00 |

**Table 4.21: Pairwise Comparison of Metagenome Species Richness between Different Regions Based on Chao1 Diversity Index. P Value Adjustment Method: Holm**

| | **Bungoma** | **Kilifi** | **Kwale** | **Siaya** |
|---|---|---|---|---|
| Kilifi | 1.00 | - | - | - |
| Kwale | 1.00 | 1.00 | - | - |
| Siaya | 1.00 | 1.00 | 1.00 | - |
| Turkana | 1.00 | 1.00 | 1.00 | 1.00 |

The NMDS with Jaccard distance was used for dimension reduction analysis (Figure 4.36). NMDs plot shows the differences between the two groups (faecal and caecal samples) according to Bray-Curtis distance. There was no clear demarcation between bacterial assemblages from the faecal and caecal samples of the studied poultry.

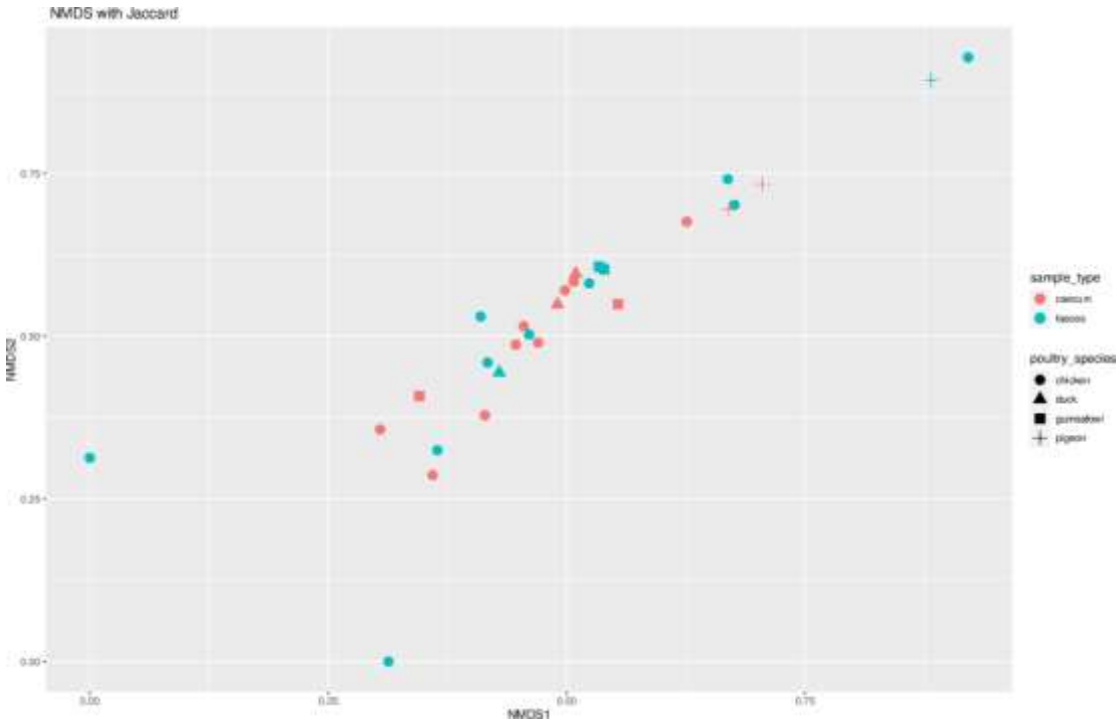


**Figure 4.36: Pairwise Comparison of Differences between Faecal and Caecal Metagenomes in the Different Poultry Using the NMDS Plot with Jaccard Distance.**

Pairwise comparison of differences between faecal and caecal metagenomes in different poultry was also done using the PCoA plot with Bray-Curtis distance. (Figure 4.37). Based on the ordination of the distance matrix generated using the Bray-Curtis complementary algorithm, a clear demarcation between bacterial assemblages from the faeces and caeca was equally not apparent along principal coordinate axis 1 (PCO1) of the PCoA plot.

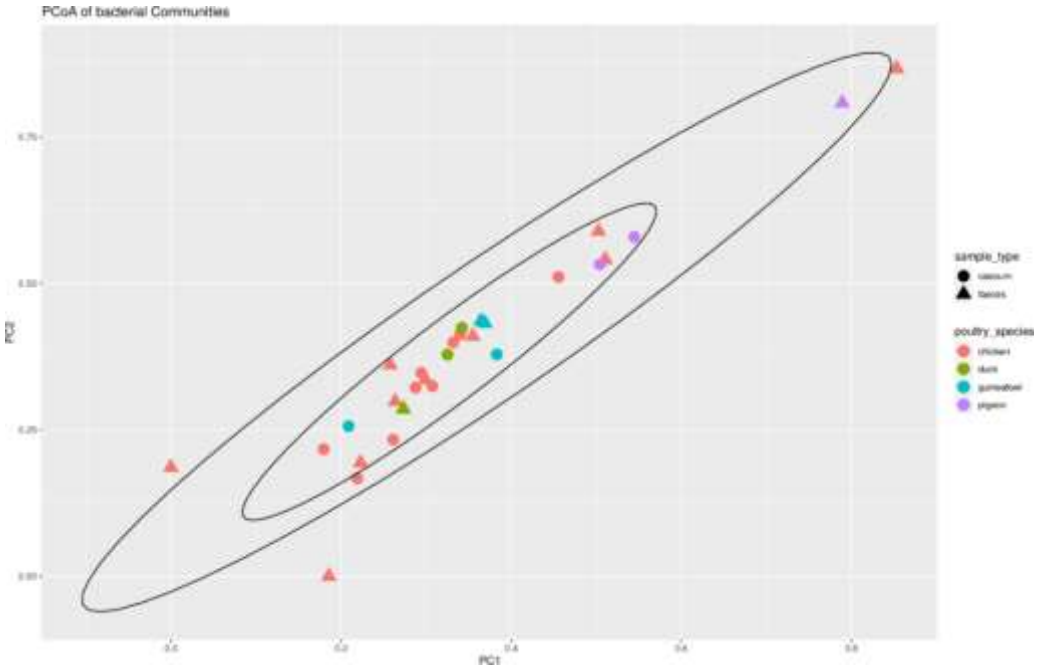


**Figure 4.37: Pairwise Comparison of Differences between Faecal and Caecal Metagenomes in Different Poultry Using the Pcoa Plot with Bray-Curtis Distance.**

### 4.4.2 Functional Annotation

Functional diversity of a microbial community can be quantified by annotating metagenomic sequences with functions (Kumar et al., 2020). Classification of an assembled metagenomic protein sequence into a protein family (function) requires searching to protein family databases. Protein coding sequences were mapped against KEGG and COG databases. Relative abundance in level 1 hits of each database was plotted as a heatmap of functional abundance for each sample (Figures 4.38 and 4.39). The KEGG pathway analysis showed that functions such as transposition, metabolism, cellular processes, and human diseases were predicted in the faecal and caecal samples. The COG pathway analysis, on the other hand, showed that cellular processes and signaling, information storage and processing, and metabolism were detected in the faecal and caecal samples.

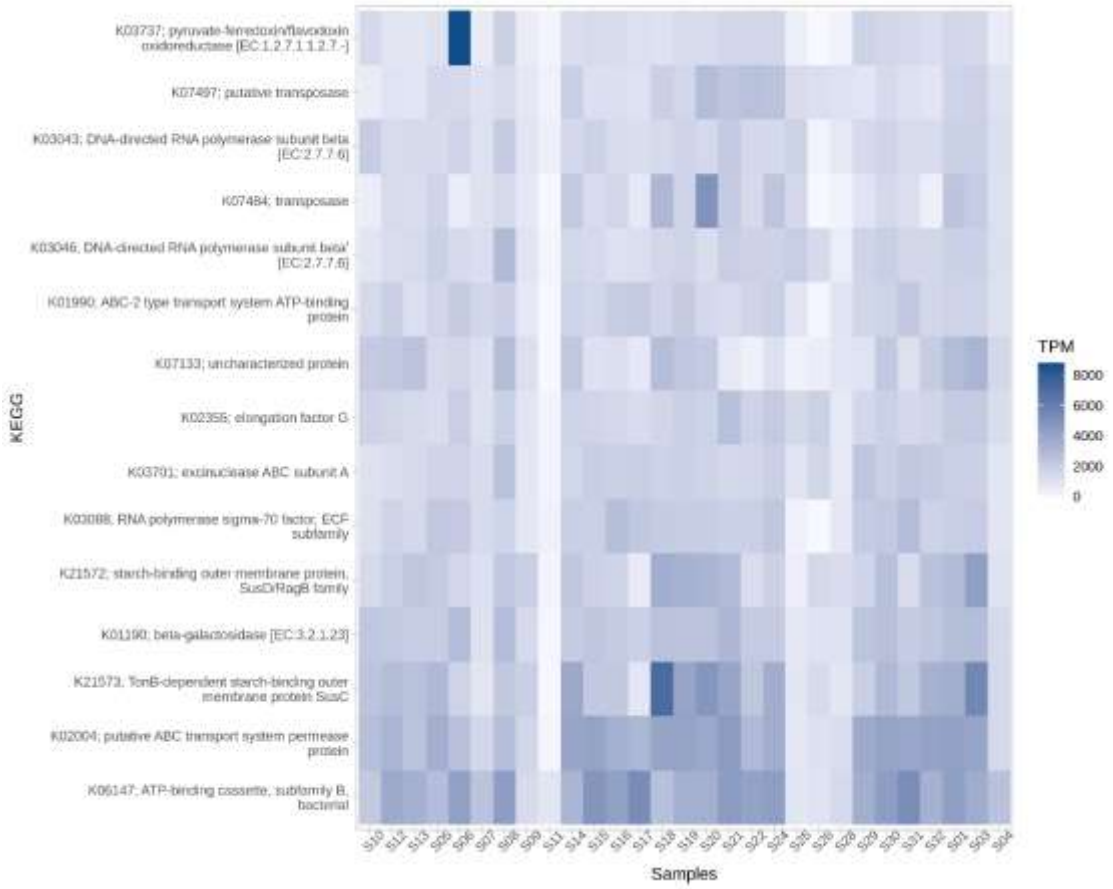


**Figure 4.38: Sample-Wise KEGG Pathway Distribution Plot Showing Functional Relative Abundance (Y-Axis) for all Samples (X-Axis) at Different Taxonomic Levels between the Two Types of Microbiomes.** Colours scale from blue (high abundance) to lavender (low abundance) represent log-transformed relative abundance. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

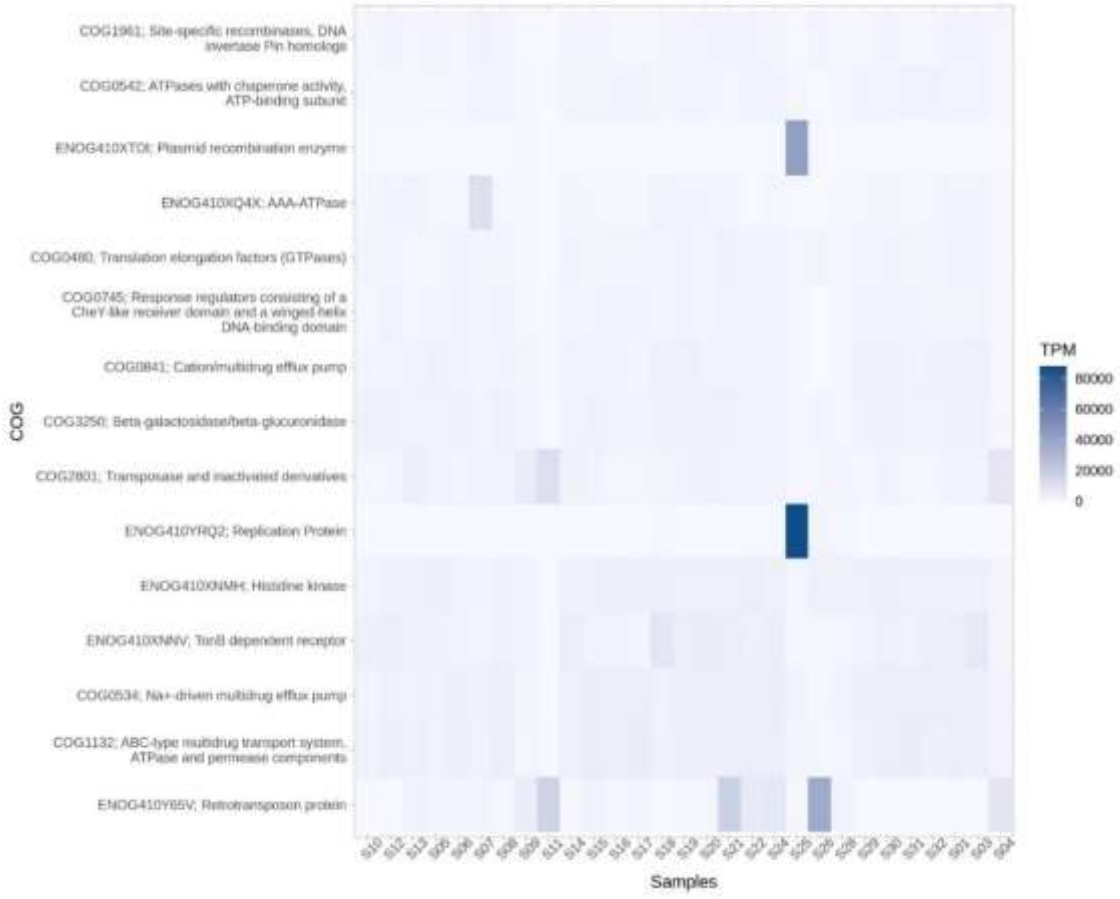


**Figure 4.39: Sample-Wise COG Pathway Functional Relative Abundance (Y-Axis) for all Samples (X-Axis) At Different Taxonomic Levels between the Two Types of Microbiomes.** Colours scale from blue (high abundance) to lavender (low abundance) represent log-transformed relative abundance. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

### 4.5 Detection of Antimicrobial Resistance Genes

The Antimicrobial Resistance Genes Database (ARDB) was used to identify ARGs in the faecal and caecal samples across all poultry. Identified ARGs were further categorized on the basis of their resistance profiles and sequence similarity. Each gene or type was annotated with information that included resistance profile and mechanism. According to abundance table of ARGs, downstream analyses were performed.

### 4.5.1 Detection of ARGs in Poultry Cloacal Samples

The major antimicrobial resistance genes found in the cloacal samples across all poultry are shown below in a bargraph and heat map (Figures 4.40 and 4.41). Several genes responsible for AMR were detected, such as those conferring resistance to beta-lactamases (*TEM116*, *TEM33*, *TEM4*, *TEM3*, and *aadA12*), tetracycline (*tetC* and *tetW*), aminoglycosides (*APH3Ib*), sulfonamides (*sul2*), and multidrug efflux pumps (*acrB*, *tolC*, and *emrR*). Beta-lactamase-resistant genes were detected in most poultry while tetracycline-resistant genes were only detected in some chicken and duck samples. Other proteins associated with AMR such as *HNS*, *CRP*, and *robA* were also identified. Ducks, guinea fowls, geese, and turkeys had the highest concentration of ARGs.

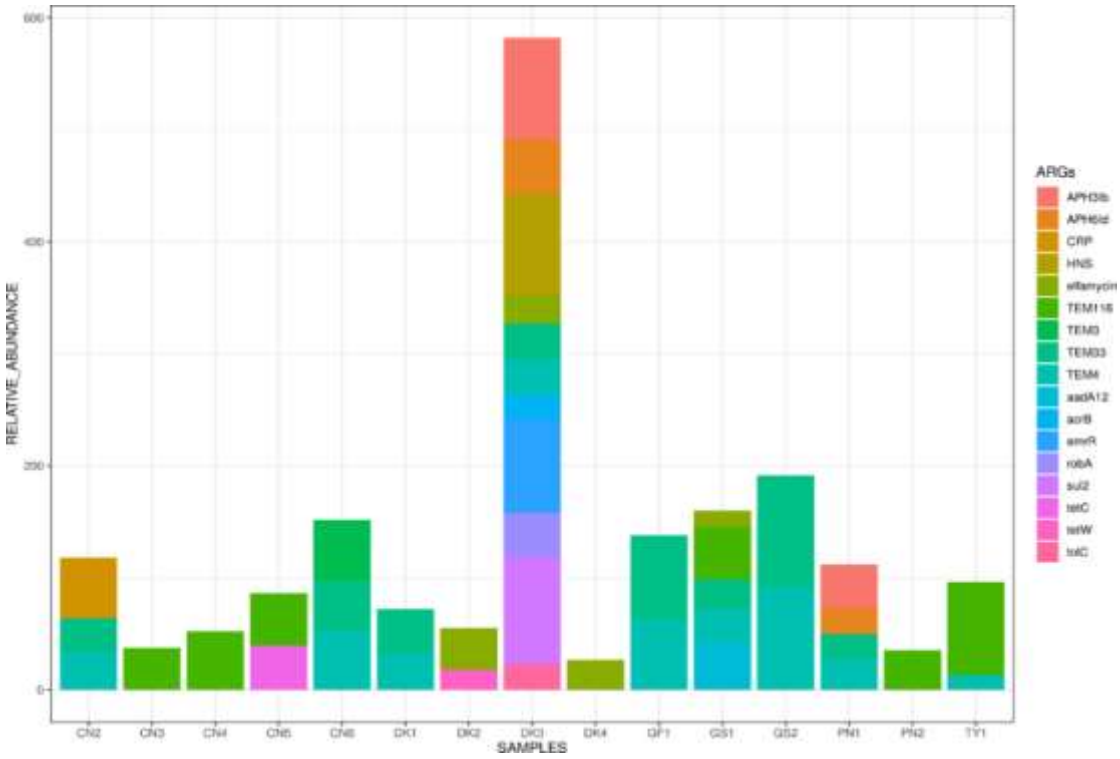


**Figure 4.40: Bargraph of Total Level of Antimicrobial Resistance Genes in the Cloacal Samples.** The stacked column chart shows relative abundances of AMR genes aggregated to corresponding ARGs (y-axis) by sample (x-axis) with the height of each bar chart relating to the relative AMR gene abundances in a sample. For each

poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

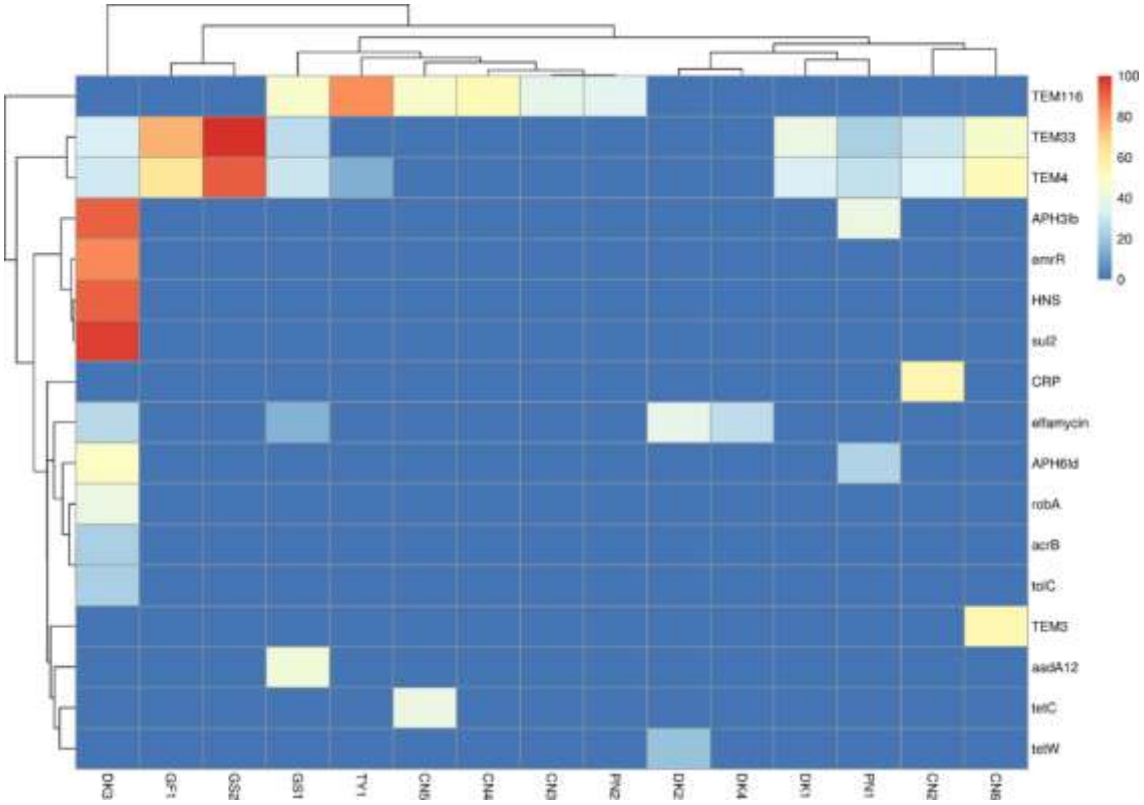


**Figure 4.41: Heatmap of Antimicrobial Resistance Gene Relative Abundances in Cloacal Samples Based on Log-Transformed Relative Abundance Values.** The color scale from red (high abundance) to blue (low abundance) represents log-transformed relative abundance, and blue (0 on a scale) means no ARGs detected. For each poultry and sample type (CN = chicken, DK = duck, GF = guinea fowl, GS = goose, PN = pigeon, TY = turkey).

### 4.5.2 Detection of ARGs in Poultry Faeces and Caeca

The major ARGs found in faecal samples are shown in Figures 4.42 and 4.43. The bargraph and heatmap show a variable abundance of ARGs between samples. The majority of the ARGs detected in most poultry were those conferring resistance to tetracycline (*tetW3*, *tetQ1*, *tetA*, *tetW1*, and *tetW5*). However, *tetQ1* and *tetW1* were

detected in most of the poultry faecal samples, implying that these are the most common tetracycline-resistant genes in poultry faeces. Other ARGs detected in some samples included those that confer resistance to β-lactamases (*bla*$_{OXA851}$), aminoglycosides (*aph6ld1* and *aph3lb1*), and sulfonamides (*sul211*), although these antibiotic classes were only detected in chicken faecal samples.

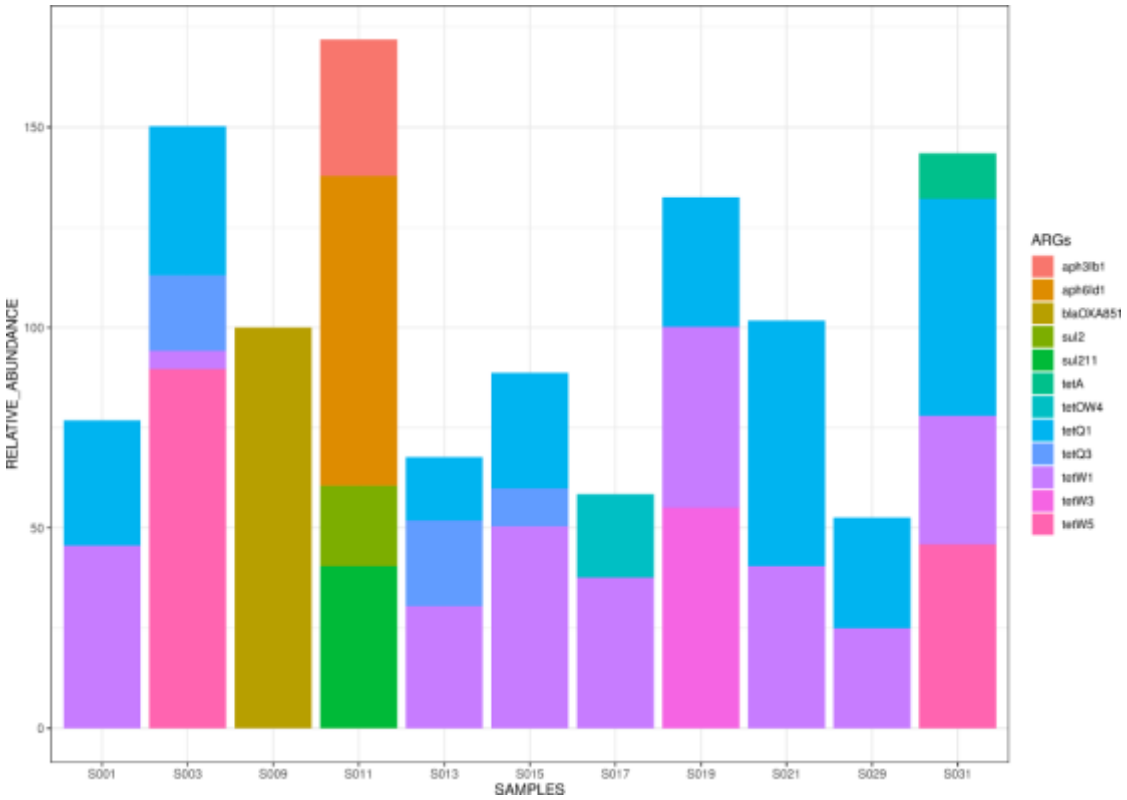


**Figure 4.42: Bargraph of Total Level of Antimicrobial Resistance Genes in the Faecal Samples.** The stacked column chart shows relative abundances of AMR genes aggregated to corresponding ARGs (y-axis) by sample (x-axis) with the height of each bar chart relating to the relative AMR gene abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

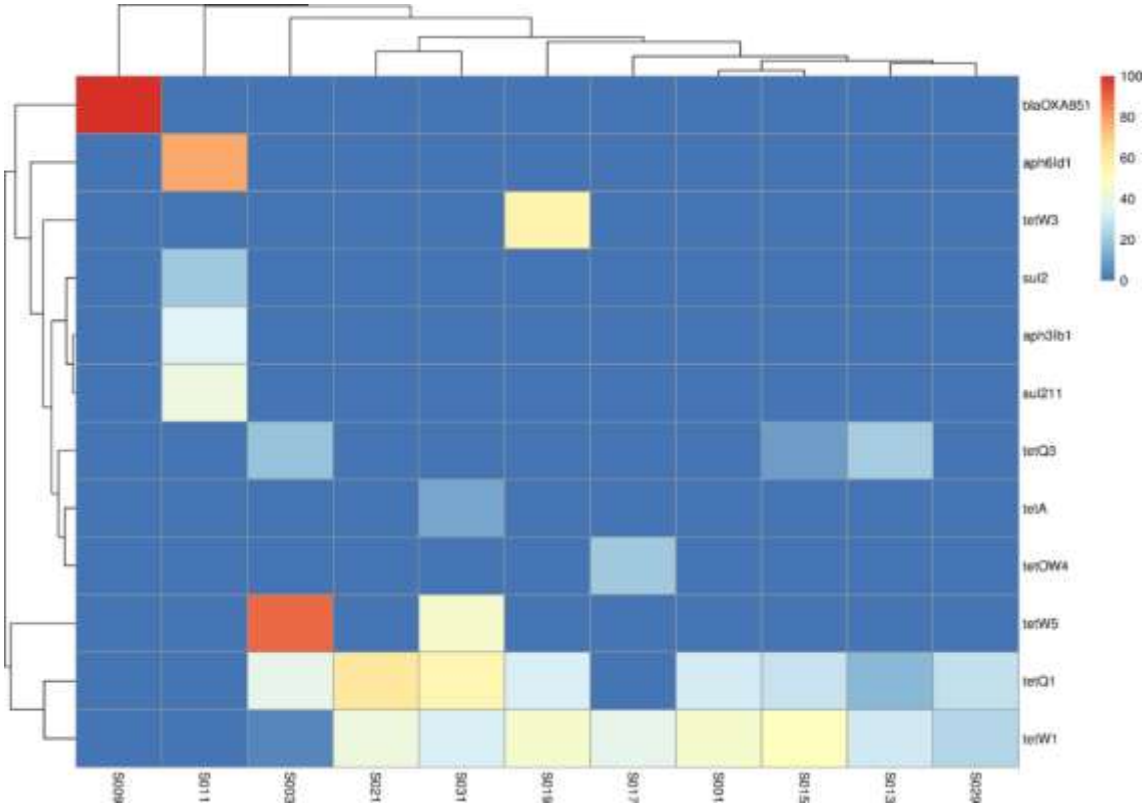


**Figure 4.43: Heatmap of Antimicrobial Resistance Gene Abundance in Faecal Samples Based on Log-Transformed Relative Abundance Values.** The color scale from red (high abundance) to blue (low abundance) represents log-transformed relative abundance, and blue (0 on a scale) means no ARGs detected. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

The major ARGs found in caecal samples are shown in Figures 4.44 and 4.45, also showing that the abundance of ARGs between samples was variable. Similar to faecal samples, genes conferring resistance to tetracycline (*tetQ1*, *tetQ2*, *tetQ3*, *tetW1*, *tetW3*, and *tetW5*) were detected in higher numbers in most of the caecal samples, with *tetQ1*, *tetW1*, and *tetW5* being common across most poultry. Other ARGs detected included those conferring resistance to β-lactamases ($bla_{OXA2091}$), sulfonamides (*sul2*), and macrolides (*ermF1*, *ermF3*, and *ermG1*). These other antibiotic classes were only detected in chicken faecal samples. Multidrug efflux pumps (*let44*) were also detected.

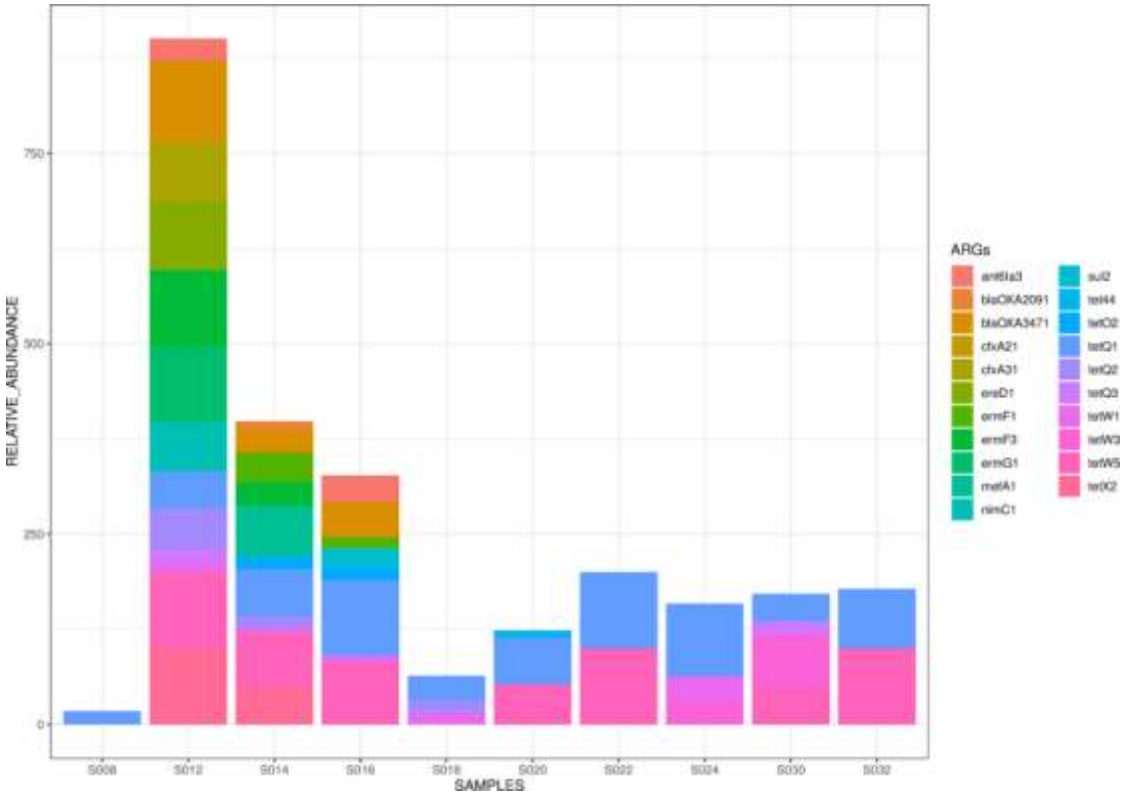


**Figure 4.44: Bargraph of Total Level of Antimicrobial Resistance Genes in the Caecal Samples.** The stacked column chart shows relative abundances of AMR genes aggregated to corresponding ARGs (y-axis) by sample (x-axis) with the height of each bar chart relating to the relative AMR gene abundances in a sample. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

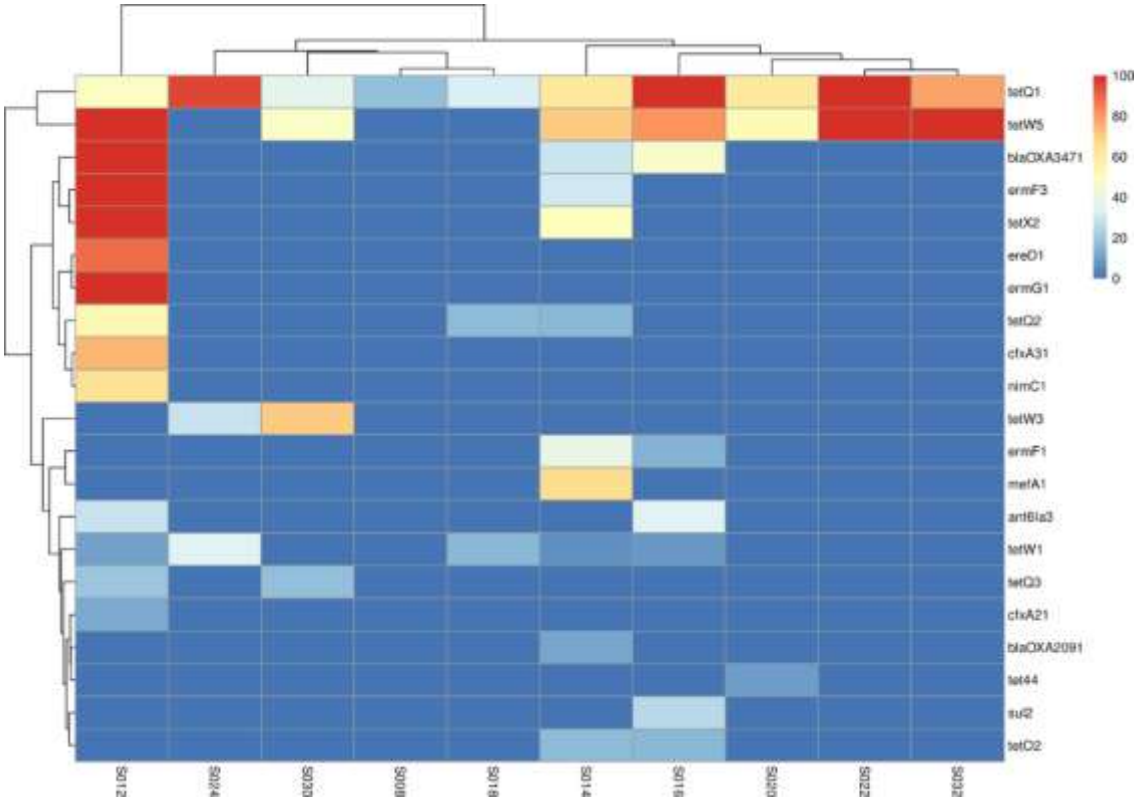


**Figure 4.45: Heatmap of Caecal Antimicrobial Gene Relative Abundance Based on Log-Transformed Relative Abundance Values**. The color scale from red (high abundance) to blue (low abundance) represents log-transformed relative abundance, and blue (0 on a scale) means no ARGs detected. For each poultry and sample type (S001-S020 = chicken, S021-S024 = duck, S025-S028 = pigeon, S029-S032 = pigeon).

# CHAPTER FIVE

# DISCUSSION

## 5.1 Introduction

This Chapter discusses and interprets the findings of the study and also highlights its significance in the immediate context of the metagenomic detection and characterization of enteric viral and bacterial pathogens, evaluation of gut microbial profiles, and determination of antimicrobial resistance genes in poultry in selected regions in Kenya.

## 5.2 Characterization of Enteric Viral Pathogens

From the 254 viral contigs detected in poultry cloacal samples, 77 viral contigs had no significant similarity to any sequences identified in Genbank, which was 30 % of the contigs that mapped to viral sequences. This was higher than the observations made by Lima et al. (2019) that the percentages of eukaryotic viral reads detected in the mal-absorption syndrome affected and healthy chicken were 22.1 % and 14.5 % respectively. The proportion of viral contigs that had no significant similarity to any sequences identified in Genbank was also higher than the proportion of unclassified sequences reported from the metagenomic analysis of the fecal virome in asymptomatic pigs in East Africa (Amimo et al., 2016).

Eleven potentially pathogenic viruses from the poultry samples were identified. Similar studies revealed the presence of pathogenic viruses in oropharyngeal swab samples (Ogali, 2020; Qiu et al., 2017). The findings of this study are also consistent with previous studies that identified these viruses as major pathogens in poultry (Kariithi et al., 2023; Umar et al., 2019). It is noteworthy that most of the chicken sold in Nairobi are transported from Western Kenya, especially Trans Nzoia, and the coastal region, including Kilifi and Kwale counties. This implies that there is continuous circulation of pathogenic viruses between backyard poultry species throughout the country, as opined by Ogali et al. (2018).

Avian IBV sequences detected compared well with the other previously described Avian IBV sequences. Avian IBV is a highly contagious avian coronavirus that causes respiratory disease in chickens, leading to economic losses in the poultry industry. Avian coronaviruses have been implicated in certain severe infections in poultry. The presence of coronaviruses is of particular interest since they have previously been associated with inter-species spill over, for instance, severe acute respiratory syndrome coronavirus (SARS-CoV) 1 and 2 and Middle Eastern respiratory syndrome coronavirus (SERS-CoV) (Ommeh et al., 2018; Vibin et al., 2020). In a study conducted in Kenya, IBV was identified as a common cause of respiratory disease in poultry (Kariithi et al., 2023). This also suggests that IBV is a significant threat to the poultry industry in Kenya and that further research is needed to understand the epidemiology and pathogenesis of the virus.

Rotaviruses (Rotavirus F and G) were also detected in high numbers in chicken samples. Although infection with this serotypes has not been considered fatal, infection from other Rotavirus serotypes can be lethal to poultry in extreme cases since it is one of the common enteric viruses that causes diarrhoea in poultry (Vibin et al., 2020). Previous studies have shown that rotavirus is one of the most common viruses detected in poultry (Spackman et al., 2010; Pinheiro et al., 2023). Interestingly, rotaviruses were also detected from samples collected in Kilifi, Kwale, Nairobi, and Trans Nzoia.

Retroviruses are a group of RNA viruses that can cause a wide range of diseases in birds, including lymphomas and leukemias. It is noteworthy that a majority of the retroviral sequences detected were harbored by ducks. Ducks are a major source of pathogens, but interestingly, they mostly remain asymptomatic even as they transmit these viruses to other vulnerable poultry, especially chickens and turkeys. Avian leucosis virus and Reticuloendotheliosis virus have previously been implicated as etiological agents of some immunosuppressive and neoplastic diseases in poultry (Zheng et al., 2022). Avian leucosis virus mainly infects chickens while Reticuloendotheliosis virus infects chickens, turkeys, and other avian species (Fadly, 1997). In addition to causing tumors, both pathogens can reduce productivity and induce immunosuppression in affected flocks (Fadly, 1997). Lymphoproliferative

disease virus, a retrovirus associated with tumors in wild and domestic turkeys, has also been described in turkey flocks in Europe, the Middle East and the United States (Thomas et al., 2015). A study conducted in Brazil also detected REV in Muscovy ducks, wild turkeys, and chickens at a relatively high prevalence rate of 16.8% (Caleiro et al., 2020). The impact of Avian leucosis virus on broiler chickens has also been reported in Malaysia (Bande et al., 2016).

Other viruses that were detected in smaller numbers include Avian HDV-like agent, turkey coronavirus, and picobirnavirus. These too have been identified in other studies, including a study conducted in Korea which identified picobirnavirus as a common virus in chicken faeces (H.-R. Kim et al., 2020). Additionally, the presence of Avian HDV-like agents in ducks has also been described (Wille et al., 2018). Studies have also shown that in humans, coinfection with HDV and Hepatitis B virus (HBV) causes more severe liver disease than is seen in individuals infected with HBV alone (Centers for Disease Control and Prevention, 2020). It is equally noteworthy that most retroviruses and Avian HDV-like agents were detected in duck samples, yet ducks are rarely affected by these retroviruses, which suggests that they may serve as major hosts, carriers, or transporters of viral pathogens, as earlier alluded by Tolf et al. (2013).

Most of the non-avian host associated viruses that were identified were likely either part of the food eaten by the poultry, or bacteriophages affecting enteric bacteria, some of which are pathogenic. For instance, tomato mosaic virus and bean pod mottle virus detected in duck and chicken samples respectively, are likely to be from the food eaten by these poultry species. Bacteriophages detected were Salmonella phage LSE7621, Salmonella phage SE11, Salmonella phage vB Sen I1, Salmonella phage oldekoll, Escherichia phage Vb, Shigella phage SSP1, and Phage NBEco001. It is interesting to note that most of the identified phages infect enteric bacteria with pathogenic potential such as *Salmonella*, *Shigella*, and *Escherichia*. *Salmonella* is associated with pullorum disease, fowl typhoid, and paratyphoid infections (Porter, 1998), while *E. coli* and *Shigella* are associated with colibacillosis, air sacculitis, and cellulitis (Matin et al., 2017). The presence of these bacteriophages therefore is a possible indicator of the kind of bacteria colonizing the poultry gut. Interestingly, all

the bacteriophages were detected in duck samples, which again strongly suggests that they may serve as major hosts, carriers, or transporters of both viral and bacterial pathogens.

### 5.3 Characterization of Enteric Bacterial Pathogens

This study reports that *Proteobacteria*, *Chlamydiae*, and *Firmicutes* were the most frequently detected in the chicken cloacal samples. These results are consistent with the findings by K. Kang et al. (2021) that reported *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* as the dominant phyla in the poultry in the hindgut and feces, although *Bacteroidetes* were detected in lower numbers in the current study. However, this study's results differ from previous observations by Yan et al. (2017) and Kumar et al. (2020), who suggested that *Bacteroidetes* and *Firmicutes* were the most abundant phyla in chickens. They also differ from the findings by Andreani et al. (2020), who found *Firmicutes* to be a proportionally more dominant phylum (~95 %) in cloacal and cecal samples of broiler chickens in Northern Ireland. However, just like Andreani et al. (2020), other phyla such as *Proteobacteria*, *Tenericutes*, *Actinobacteria*, and *Bacteroidetes* were detected in lower taxa numbers. It should be noted that the difference between this study's findings and those of other authors (Yan et al., 2017; Kumar et al., 2020) could be due to the differences in environmental and agroclimatic conditions. Furthermore, their investigations were based on the general microbial profiles in the caecum of chicken, while the present study specifically considered the bacterial communities of pathogenic potential in the cloacal swabs of several poultry.

The results also showed that *Desulfovibrio*, *Gallibacterium*, and *Mycoplasma* were the most frequently detected genera in the cloacal samples across the poultry. In contrast, other studies showed that *Lactobacillus*, *Lachnoclostridium*, *Clostridium*, and *Bacteroides* were the dominant genera in the cecum, cloaca, and faeces (K. Kang et al., 2021), while enterobacteria, lactobacilli, and enterococci were found to dominate the small intestines of chickens in Malaysia (Mohd Shaufi et al., 2015). On the other hand, *Lactobacillus* and *Bacteroides* were predominant in the small intestines of chickens in China (Wei et al., 2013). Another study by Schreuder et al.

(2021) found that *Romboutsia, Gallibacterium*, and *Fusobacterium* were the most abundant across all samples, which equally contradicted the findings of this study. Most of the bacteria detected in the current study are potentially pathogenic.

At the species level, the hierarchical cluster maps revealed that *Chlamydia ibidis, Gallibacterium anatis, Escherichia coli, Avibacterium paragallinarum*, *Mycoplasma gallinaceum*, *Streptococcus suis*, and *Weissella confusa* were the most frequently detected bacterial species in the cloacal samples. Avian pathogenic *Escherichia coli* (APEC) causes colibacillosis, which is a severe respiratory and systemic disease in chickens (Alber et al., 2020), while *Chlamydia* infection in birds typically result in respiratory, ocular, and enteric symptoms, sometimes with fatal outcome, although asymptomatic, latent infections are also common (Z. Li et al., 2020). *Streptococcus* species are considered a part of the normal flora in poultry, with infections resulting from *Streptococcus* occurring secondary to other primary infections. These infections can be acute or subacute/chronic forms due to septicemia, although they can be successfully treated. However, it is a zoonotic agent that causes severe disease in humans and is a major pig pathogen worldwide (Nhung et al., 2020). The role of *Gallibacterium anatis* and *Avibacterium paragallinarum* as etiologic agents of bacterial diseases in poultry has also been reported (El-Adawy et al., 2018; Mei et al., 2020). *Weisella confusa*, on the other hand, has been proposed as a good candidate for the development of novel direct-fed microbial products (Sturino, 2018).

It should be noted however, that the comparison of OTUs and taxonomic composition between the current study and other reported studies may be affected by approaches adopted in conducting the study (Mohd Shaufi et al., 2015). Other factors such as environment, treatment, feed additives, antibiotics, age, horizontal gene transfer, hygiene level, diet, poultry species, and agroclimatic considerations may also affect the poultry gut microbiome composition (Mohd Shaufi et al., 2015). PCoA and NMDS plots showed no clear demarcation between bacterial communities among the poultry under study. This study's findings are consistent with observations made by K. Kang et al. (2021) who observed that samples from the caecum clustered with those from the cloaca in microbial structure.

## 5.4 Analysis of Poultry Microbiome

This study reports that *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the most frequently detected in both faecal and caecal samples for the poultry under study, which is similar to previous observations that reported the abundance of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* in the poultry gut metagenomes (Yan et al., 2017; Bhogoju et al., 2018; Kumar et al., 2020; K. Kang et al., 2021). The results are also consistent with previous studies that reported the presence of *Archaea* (such as *Euryarchaeota*) in the poultry gut, although at lower abundances (Yeoman et al., 2012).

This study also identified *Bacteroidaceae*, *Oscillospiraceae*, *Methanobacteriaceae*, *Lachnospiracaea*, *Clostridiaceae*, *Prevotellaceae*, *Synergistaceae*, *Chlamydiacaea*, and *Enterobacteriaceae*, among others, in both faecal and caecal samples. Bhogoju et al. (2018) reported that members of the family *Lachnospiracaea* (belonging to the phylum *Firmicutes* and class *Clostridia*) were identified abundantly in the digestive tracts of animals, with several members helping in producing butyric acid, which is important for both microbial and host epithelial cell growth. Additionally, Scheperjans et al. (2015) posited that *Prevotellaceae* (belonging to the phylum *Bacteroidetes*) is a family of bacteria that is known to help in breaking down proteins and carbohydrates and commonly colonizes the gut of animals. Research is also ongoing to establish the relationship between members of the *Prevotellaceae* family and Parkinson's disease (Scheperjans et al., 2015) . Members of the family *Enterobacteriaceae* are also regularly found in animal guts, mostly as commensal organisms. However, some, including *Salmonella* and *Escherichia*, are pathogenic.

*Bacteroides*, *Methanobrevibacter*, *Phocaeicola*, *Candidatus Adamsella*, *Mediterranea*, and *Pseudoflavonifractor* were the most frequently detected genera in poultry faecal samples while the dominant genera in the caecal samples were *Bacteroides*, *Methanobrevibacter*, *Chlamydia*, *Pseudoflavonifractor*, *Elusimicrobium*, *Candidatus Alloclostridium*, *Faecalibacterium*, and *Prevotella*. On the contrary, enterobacteria, lactobacilli, and enterococci were observed to dominate the chicken gut in Malaysia (Mohd Shaufi et al., 2015), while *Lactobacillus* and

*Bacteroides* were predominant in the small intestines of chickens in China (Yan et al., 2017). *Bacteroides* are important commensals in the poultry gut as they have been implicated in the degradation of essential complex carbohydrates and also produce short-chain fatty acid (SCFA) (Fan et al., 2023). *Methanobrevibacter* has equally been reported to be the predominant archaeal genus in the poultry gut, with *Methanobrevibacter woesei* being the most prolific of the archaeal domain in poultry (Yeoman et al., 2012). The roles of *Chlamydia*, *Pseudoflavonifractor*, *Elusimicrobium*, *Faecalibacterium*, and *Prevotella* as important commensal microorganisms in poultry have been reported (Scheperjans et al., 2015; Tabashsum et al., 2020; Wang et al., 2020; Khan & Chousalkar, 2021; Marchino et al., 2022).

PCoA and NMDS plots showed no clear demarcation between bacterial assemblages from the faecal and caecal samples across the poultry under study. However, previous studies reported distinct metagenome structures in poultry (Kumar et al., 2020; B. Li et al., 2015; Zhao et al., 2013). As explained earlier, this could be due to other environmental factors not related to host and microbial community interactions.

The KEGG pathway analysis showed that functions such as metabolism, cellular processes, and human diseases were predicted in the faecal and caecal samples. The COG pathway analysis, on the other hand, showed that cellular processes, information storage and processing, and metabolism were detected in the faecal and caecal samples. These findings are consistent with the observations made by Kumar et al. (2020), who reported that metabolism, genetic information processing, cellular processes, human diseases, and organismal systems were the dominant functions predicted at level one in the KEGG pathway analysis.

### 5.5 Analysis of Antimicrobial Resistance

#### 5.5.1 Detection of ARGs in the Poultry Cloaca

Using whole genome sequencing and metagenomics, a summary of the antimicrobial resistance genes present in the analyzed poultry samples is presented. Several genes responsible for antimicrobial resistance were detected in cloacal samples, with the most predominant genes conferring resistance to β-lactamases. Other genes detected

were those conferring resistance to tetracycline, aminoglycosides, sulfonamides, and multidrug efflux pumps. This study corroborates the observations by Ndukui et al. (2022) who found a high prevalence of extended-spectrum beta-lactamase (EBSL)-producing *Escherichia coli* in poultry in Kenya. Ducks, guinea fowls, geese, and turkeys had the highest concentration of antimicrobial resistance genes, underscoring the importance of these poultry as disseminators of antimicrobial resistance. Of major concern is that a combination of these antimicrobial resistance genes is expected to confer significantly high resistance to a wide range of antibiotics, including beta-lactams, aminoglycosides, and tetracyclines. These drug classes are the mainstream antibiotics that are indicated for the prophylaxis and treatment of bacterial infections in humans and animals (Kobayashi et al., 2014).

### 5.5.2 Detection of ARGs in the Poultry Faeces and Caeca

Several antimicrobial resistance genes were detected in faecal samples, with the most predominant genes conferring resistance to tetracycline, with *tetQ1* and *tetW1* being detected in most of the poultry faecal samples, implying that these are the most common tetracycline-resistant genes in poultry faeces. Other antimicrobial resistance genes detected in some samples included those that confer resistance to β-lactamases, aminoglycosides, and sulfonamides, although these antibiotic classes were only detected in chicken faeces, suggesting the threat posed by antimicrobial resistance to indigenous backyard chickens.

Similarly, the major antimicrobial resistance genes found in caecal samples were those conferring resistance to tetracycline. Other ARGs detected included those conferring resistance to β-lactamases, sulfonamides, and macrolides. These other antibiotic classes were only detected in chicken caecal samples. In a similar study investigating AMR in Ethiopian backyard chickens, Kumar et al. (2020) reported that the most predominant ARGs detected were those conferring resistance to tetracycline. These results are also consistent with the data from other metagenomics studies on poultry (Skarżyńska et al., 2020; K. Kang et al., 2021) which underscore the threat of AMR to indigenous backyard poultry production in Kenya.

The abundance of genes conferring resistance to tetracycline could probably be a result of ongoing selective pressure in the environment, as tetracyclines are the most widely used antimicrobial class in veterinary medicine and horticulture (Skarżyńska et al., 2020). The authors also noted that the other AMR genes that are commonly used in farm animals belong to the antibiotic classes that are quite crucial to humans, such as macrolides, aminoglycosides, and beta-lactams. It should be noted, however, that although the presence of ARGs does not necessarily mean resistance of a particular microorganism to the corresponding antimicrobial agent, it nonetheless increases the risk of AMR development. This study's findings underscore the need to understand bacterial pathogens affecting poultry and also find ways to control the inappropriate use of antimicrobials since ARGs can be transmitted from poultry to humans by consuming contaminated poultry products.

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATIONS

## 6.1 Introdution

This Chapter presents Conclusions and Recommendations drawn from the study.

## 6.2 Conclusion

This study characterized enteric viral and bacterial pathogens in Kenyan poultry, evaluated the poultry gut microbial community profiles, and detected antimicrobial resistance genes associated with these microorganisms in poultry raised under free-range production systems in Kenya. The findings of this study have been reported and are expected to provide a repository of information that will guide policy makers on the proper management of enteric viral and bacterial pathogens in poultry, provide a better understanding of gut microorganisms, and offer alternatives to antimicrobial use in order to control antimicrobial resistance in poultry (Appendix II and III).

### 6.2.1 Characterization of Enteric Viral Pathogens

The present study has demonstrated the presence of several enteric viruses of pathogenic potential, especially IBV, other coronaviruses, rotaviruses, and retroviruses. This study therefore rejects the null hypothesis that there are no enteric viral pathogens in Kenyan poultry, as many significant viruses of pathogenic potential were detected. The identification of IBV, rotaviruses, and retroviruses as major pathogens in poultry suggests that these viruses are significant threats to the poultry industry and that further research is needed to develop effective control strategies.

### 6.2.2 Characterization of Enteric Bacterial Pathogens

The study also investigated poultry enteric bacterial pathogens from different geographical locations in Kenya. The results indicate the presence of many pathogenic bacteria in the different poultry species studied, especially those

belonging to the phyla *Proteobacteria, Chlamydiae,* and *Firmicutes*. The specific enteric bacteria of pathogenic potential detected were *Chlamydia ibidis, Gallibacterium anatis, Escherichia coli, Avibacterium paragallinarum, Mycoplasma gallinaceum, Streptococcus suis,* among others. These bacteria have been implicated as aetiologic agents for several poultry infections. Hence the null hypothesis stating that there are no enteric bacterial pathogens in Kenyan poultry is also rejected.

### 6.2.3 Analysis of Poultry Microbiome

Additionally, this study compared the faecal and caecal microbiota of chickens, ducks, pigeons, and guinea fowls raised under free-ranging conditions. The results indicate the presence of many commensal microorganisms in the poultry studied. *Bacteroides, Methanobrevibacter, Phocaeicola, Candidatus Adamsella, Mediterranea,* and *Pseudoflavonifractor* were the most frequently detected genera in poultry faecal samples. The dominant genera in the caecal samples across the poultry species were *Bacteroides*, *Methanobrevibacter*, *Chlamydia*, *Pseudoflavonifractor*, *Elusimicrobium, Candidatus Alloclostridium*, *Faecalibacterium*, and *Prevotella*. These bacterial and archaeal taxa are important comensal gut microbiota. Comparisons of species richness between the different poultry were also tested to determine if they differed significantly. The results showed that there are no significant differences in microbial species richness between the different poultry and sample types. The study therefore does not reject the null hypothesis that there are no significant differences in poultry enteric microbial community profiles. This study improves our understanding of the poultry gut microbiome and is a valuable resource for possible application of probiotics in poultry production.

### 6.2.4 Analysis of Antimicrobial Resistance

Finally, this study also reports the abundance of ARGs that confer resistance to β-lactamases, aminoglycosides, and tetracycline in the cloacal swabs of most of the poultry analyzed. Genes conferring resistance to tetracycline were also found to be the most abundant in the faecal and caecal samples, raising concern about the dangers associated with the inappropriate and unregulated use of this antibiotic for treating poultry. Ducks, guinea fowls, geese, and turkeys had the highest

concentration of ARGs, underscoring the importance of these poultry as disseminators of AMR. Of major concern is that a combination of these ARGs is expected to confer significantly high resistance to a wide range of antibiotics, including beta-lactams, aminoglycosides, and tetracyclines. These drug classes are the mainstream antibiotics that are indicated for the prophylaxis and treatment of bacterial infections in humans and animals. The study therefore rejects the null hypothesis that enteric pathogens in Kenyan poultry are not resistant to antimicrobial agents. The ARG data generated in this study provides a valuable indicator of the use of antimicrobials in poultry by smallholder indigenous backyard poultry farmers in Kenya.

### 6.3 Recommendations

- The present study has demonstrated the presence of several viruses that have previously been identified in cloacal swab samples in poultry. However, further research is recommended to determine the proportion of the detected viruses that is commensal vis avis the pathogenic viruses.
- The results also indicate the presence of many commensal and potentially pathogenic microorganisms in the cloacal, faecal and caecal samples of the diferent poultry species studied. The study recommends further work that compares metagenomes of poultry raised in both free range and controlled conditions to help assess the impact of the free-range environments on microbial communities of poultry.
- Finally, the study reports the abundance of ARGs that confer resistance to β-lactamases, aminoglycosides, and tetracycline in the cloacal swabs, faeces, and caeca of most of the poultry analyzed. Further research is recommended to investigate the specific bacterial species associated with these antimicrobial resistant genes.

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## APPENDICES

### Appendix I: Microbiome Assessment of Local Poultry Populations in Kenya

Sample ID_____ Date_____

Farmer's name_____

County _____ Sub County_____

Village_____ GPS_____

Sex: Female ☐ Male ☐

Poultry species reared

Chicken ☐ Guinea fowl ☐ Quail ☐ Duck ☐ Pigeon ☐

Other_____

Number of poultry_____ Poultry weight_____

Source of poultry_____

What type of management system do you practice?

Extensive ☐ semi-intensive ☐ intensive ☐ others, specify_____

Condition of the poultry structures if any_____

Are the poultry infested by ectoparasites? Yes ☐ No ☐

Do you give commercial feed to your poultry?

Yes ☐ No ☐

Which commercial feeds do you give? _____

How frequently do you feed your birds daily? _____

How many eggs are laid per clutch? _____

Chicken internal temperature _____

Outside temperature_____

Threats faced by indigenous poultry_____

_____

## Appendix II: Publications from this Research Work

1. **Panyako, P.M.**, Ommeh, S.C., Kuria, S.N., Lichoti, J.K., Musina, J., Nair, V., Nene, V., Munir, M., and Oyola, S.O. (2024). Metagenomic Characterization of Poultry Cloacal and Oropharyngeal Swabs in Kenya Reveals Bacterial Pathogens and Their Antimicrobial Resistance Genes. *International Journal of Microbiology*, Volume 2024, Article ID 8054338, 18 pages. https://doi.org/10.1155/2024/8054338.
2. **Panyako, P.M.**, Ommeh, S.C., Kuria, S.N., Lichoti, J.K., Musina, J., Nair, V., Nene, V., Oyola, S.O., and Munir, M. (2023). Metagenomic Characterization Reveals Virus Coinfections Associated with Newcastle Disease Virus among Poultry in Kenya. *Journal of Basic Microbiology*, 1–14. https://doi.org/10.1002/jobm.202300390.

**3.** Panyako, P.M., Lichoti, J.K., and Ommeh, S.C. (2022). Antimicrobial Drug Resistance in Poultry Pathogens: Challenges and Opportunities. *Journal of Agriculture, Science & Technology*, 21(1):62-82. https://ojs.jkuat.ac.ke/index.php/JAGST.

## Appendix III: Conferences Where this Work Was Presented

1. International Society of Animal Genetics (ISAG) Virtual Conference (July 26-30, 2021).
2. 5th National Museums of Kenya Biennial Science Conference (November 7-9, 2018).